Biological effects in human erythrocytes in vitro exposed to xenobiotics : influences by metabolizing systems from rat liver

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Biological effects in human erythrocytes *in vitro* exposed to xenobiotics

Influences by metabolizing systems from rat liver

PROEFSCHRIFT

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"Facts do not cease to exist because they are ignored" *(A. Huxley)*

Aan mijn ouders

Contents

Chapter 1

General introduction

Introduction

Human beings are exposed to a great variety of compounds of foreign origin (xenobiotics), that are either of natural origin or synthetized by man and are present in food, the environment or more specifically in the work environment [60]. To estimate the nature and extent of health risks imposed by xenobiotics, toxicologists usually apply series of test systems that are mainly based on (or derived from) animal tissues. For the usual purpose —estimation of human risk— data obtained from *in vitro* and *in vivo* animal studies have to be extrapolated to man. The uncertainties caused by *in vitro* to *in vivo* extrapolation or from the extrapolation from animal to man are taken into account by the introduction of safety factors. Consequently, there is a need for methods or test systems that approach the human *in vivo* situation as closely as possible. Test systems based on *human tissue* may be a valuable tool to improve the validity of the extrapolation to man. Moreover, they can give insight into mechanistic aspects of adverse effects in man [11].

Human blood is a biological material which is relatively readily available. It is composed of *plasma* and *cells*. Elements in plasma and/or cells may be affected as a result of exposure to xenobiotics. Since blood comes in close contact with organs during its circulation through the arteries and veins, metabolites generated and excreted by the organs may also affect plasma and/or cells. *In vitro* incubations using human erythrocytes may be a valuable tool in toxicological research since biological effects in erythrocytes caused by xenobiotics can easily be evaluated. The advantages of *in vitro* incubations over *in vivo* toxicological research are (i) compounds which cannot be tested *in vivo* because of ethical reasons can be tested (ii) higher concentrations of the test compound can be tested (iii) the effect of a compound on erythrocytes can be studied without interference of kinetics and/or metabolic activity of other tissues and/or organs. In addition to the direct action of foreign compounds on erythrocytes, xenobiotics may also be metabolised by erythrocytes since these cells also contain metabolic activity themselves [8,23,41,66,78,80,109]. Erythrocytes may also be affected by products

of metabolic activity of other cells. *In vitro* co-incubation of erythrocytes and other cells can be used in toxicological research to study the interaction between erythrocytes and the other cells. Since the liver is the organ with the highest biotransformational activity, methods were developed in which erythrocytes are co-incubated with liver derived tissue.

Erythrocytes contain an interesting amount of biological parameters that may be affected by xenobiotics or their metabolites. An overview of biological parameters in erythrocytes that were found to be affected in man when exposed to different xenobiotics is given in Table 1.1. Since glutathione concentrations can be affected both by electrophilic and oxidative stress, glutathione depletion was the biological effect parameter which was studied most intensively in this thesis. Oxidation of hemoglobin and lipid peroxidation were evaluated because they are a result of oxidative stress. Glutathione S-transferase activity changes can also be considered as a result of oxidative and electrophilic stress. For this reason this parameter was also tested in this thesis.

Human erythrocytes

General aspects

Red blood cells transport oxygen from the lungs to the other tissues and carbondioxide in the opposite direction. The largest fraction of the red blood cell pool consists of mature erythrocytes (>99 %). These cells do neither contain a nucleus nor organelles. Their direct predecessors, reticulocytes, do still contain some basophilic material originating from organelles [40]. Erythrocytes circulate in the body about 120 days before being removed, usually by the spleen [40].

Erythrocytes are exposed to oxidative stress, as a consequence of their oxygen carrying function, and to nutrients and metabolic waste products because of the central role blood plays in their transport. Erythrocytes also come in close contact with xenobiotics directly at the porte d'entrées (i.e. the lungs, the gastro intestinal tract and the skin). Moreover, erythrocytes are also in close contact with cells and organs that may transform non-toxic compounds into toxic metabolites (e.g. the liver). The uptake of these compounds into the erythrocyte depends on the lipid-water partition coefficient, which in turn is dependent on the charge characteristics and the molecular dimensions of a compound [113]. Since erythrocytes possess detoxifying capacity for both electrophilic compounds and reactive oxygen species or products generated by these reactive oxygen species (see section Biochemistry), erythrocytes may play an important role in the protection of organs against these stressors.

To keep their cellular integrity, erythrocytes must protect themselves against (i) oxidants, which may peroxidize the lipid bilayer or oxidize proteins and (ii) elec-

Table 1.1: Examples of biological events that can occur in erythrocytes as a result of *in vivo* exposure of humans to xenobiotics and several parameters allied to these biological events.

Biological event	parameter	Source
membrane deformation	spectrin-hemoglobin crosslinking	oxidative damage [96]
protein adduct formation	hemoglobin adducts	aniline [65, 77] ethylene oxide [17, 30] propylene oxide [79] cigarette smoke [108, 101]
changes in enzyme activities	pyrimidine 5'-nucleotidase	lead [107]
	acetylcholinesterase	organophophates [43, 45] carbamates [43]
	glutathione S-transferase	1,3 dichloropropene [16] creosote [26] rubber workers [58] coal workers' pneumoconiosis [27] running [28]
(Antioxidant enzymes)	glutathione peroxidase	coal mine dust [25]
	catalase	lead [103]
	superoxide dismutase	lead [103]
glutathione depletion	glutathione concentration	1,3 dichloropropene [16] creosote [26] coal workers' pneumoconiosis [25] running [28]

trophiles, which may conjugate to thiol-groups of proteins. Erythrocytes contain several, usually enzyme-regulated, protective mechanisms to minimize injurious effects that result from oxidative products and toxic chemicals. Knowledge of biochemical pathways is necessary to understand the design and interpret the results of experiments as presented in this thesis; therefore biochemical aspects of the parameters that are of concern in this thesis will be made in the following paragraphs.

Biochemistry

Erythrocytes need energy and reducing equivalents (i.e. suppliers of electrons) to perform detoxifying reactions. As erythrocytes do not contain mitochondria, energy (ATP) and NADH are supplied by glycolysis [102]. $NADP^+$ is reduced to NADPH in the hexose monophosphate shunt [102]. Under normal conditions, 11 % of the glucose phosphorylated by hexokinase into glucose 6-phosphate, passes through the hexose monophosphate shunt. During oxidative stress, this can increase to 92 % [2, 76].

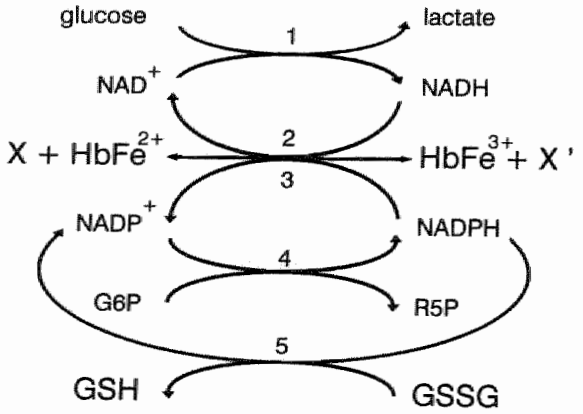

Figure 1.1: Mechanisms to keep erythrocyte hemoglobin in the reduced state, catalyzed by the enzyme(s) 1) of the glycolysis 2) NADH cytochrome b_5 reductase 3) NADPH flavin reductase 4) of the hexose monophosphate shunt and 5) glutathione reductase. X is a compound that is able to oxidise reduced hemoglobin, with subsequent formation of X'.

Ferrous hemoglobin can autoxidise into ferric hemoglobin in an environment saturated with oxygen [10]. Since the oxygen carrying function of erythrocytes is confined to ferrous hemoglobin, it must be kept in this reduced state (oxidized hemoglobin $<$ 1 % of total hemoglobin [106]). Erythrocytes possess two enzymes able to reduce oxidized hemoglobin, NADH cytochrome b_5 reductase and a NADPH flavin reductase (Figure 1.1) [87, 115, 116]. These enzymes use NADH and NADPH respectively to reduce oxidized hemoglobin. NADH cytochrome b_5 reductase is the most active enzyme, but at impaired cellular integrity, the Ca^{2+} concentration increases which inhibits NADH cytochrome b_5 reductase activity and activates NADPH flavin reductase activity [87]. Increased NADPH flavin reductase activity is expected to be of minor importance *in vivo* since an increase of the Ca^{2+} concentration in erythrocytes will probably affect the integrity of the cells with subsequent sequestration by the spleen. However, in *in vitro* incubations in which the oxidation of hemoglobin by a xenobiotic is tested, the

use of impaired erythrocytes may lead to an increase in NADPH flavin reductase activity. In this way NADPH flavin reductase reduces the effect of an oxidative compound on the formation of oxidized hemoglobin, that was expected as a result of the inhibition of NADH cytochrome b_5 reductase alone.

Reduced glutathione (GSH) protects human erythrocytes against oxidative and/or electrophilic stress. As a consequence, the GSH concentration in erythrocytes can be affected. For example, erythrocyte GSH was shown to be decreased in *in vitro* incubations with methyl chloride, methyl bromide and methyl iodide [41] and as a consequence of *in vivo* exposure to dichloropropene [16]. Beside the protective effect of GSH on erythrocytes themselves, other cells may also be protected by erythrocyte GSH. Injury of lungs caused by *in vivo* exposure of rats to hyperoxic conditions, was diminished after insufflation of erythrocytes into the trachea [4]. It was found that the glutathione redox system in erythrocytes and not superoxide dismutase or catalase, was able to protect the lungs of these rats [44].

The following section will give an overview of glutathione related biochemical reactions that take place in the erythrocyte after oxidative or electrophilic stress. In that section reference will be made to following paragraphs in which glutathione and glutathione dependent enzymes will be discussed.

Overview of GSH biochemistry in relation to oxidative and electrophilic stress

The viability of erythrocytes is dependent on the degree of exposure to stressors and the ability of the cell to cope with these stressors. Important stressors that may impede cellular functions of the erythrocyte are oxidative stress and exposure to electrophiles. GSH is a tripeptide containing cysteine which has a reactive thiol group with nucleophilic and reductive potency (see section Glutathione) and it plays an important role in the homeostasis of a cell as it (i) protects cells against oxidative challenge and (ii) detoxifies xenobiotics by conjugation [110]. These reactions are often catalyzed by GSH dependent enzymes. An overview of GSH dependent reactions that may take place in erythrocytes is given in Figure 1.2.

Depletion of glutathione (GSH or total glutathione (GT), which is the sum of both reduced glutathione (GSH) and oxidized glutathione (GSSG)) was the main biological effect parameter tested in this thesis. GSH can be affected as a result of a *chemical reaction* between GSH and (i) reactive oxygen species, which leads to oxidation of GSH, or (ii) electrophiles resulting in the generation of GSH conjugates. The latter reaction can also be catalyzed by glutathione S-transferase (GST) activity, in which an electrophile is conjugated to GSH (Figure 1.2). GSH bound in this way is not available to the cell anymore as it concerns a covalent bond. The GSH conjugates are also removed from the erythrocyte (see section Glutathione S-transferase).

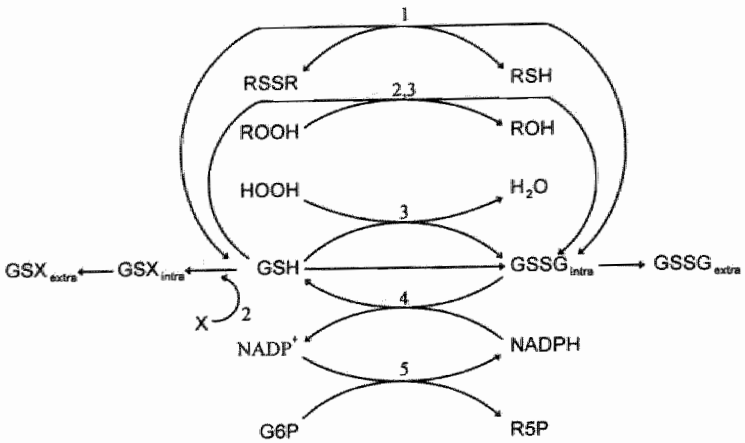

Figure 1.2: Glutathione and glutathione dependent reactions catalyzed by 1) thioltransferase 2) glutathione S-transferase 3) glutathione peroxidase 4) glutathione reductase 5) enzymes of the hexose monophosphate shunt.

Products of oxidative stress, lipid peroxides and disulfides, can be reduced by means of the action of GSH-dependent enzymes with concomitant oxidation of GSH (Figure 1.2). Glutathione peroxidase (GPx) is able to detoxify both H_2O_2 and lipid peroxides (see section Glutathione peroxidase). Lipid peroxides can also be reduced by glutathione S-transferase (GST)(see section Glutathione S-transferase), which also uses GSH as reducing equivalent. In contrast to GPx, GST is not able to detoxify hydrogen peroxide. Disulfides between proteins or between proteins and GSH can be reduced by the action of thioltransferase (TT) with concomitant oxidation of GSH (see section Thioltransferase). Since the erythrocyte must keep its reduced state, oxidized glutathione must be reduced. At normal physiological conditions, GSSG is reduced into GSH by glutathione reductase (GR) at the expense of NADPH (see section Glutathione reductase). In the erythrocyte, NADPH is generated in the hexose monophosphate shunt of glycolysis. When the reductive capacity of the erythrocyte (in this case the NADPH/$NADP^+$ ratio) is not high enough, erythrocyte GSSG will be released from the cell (see section Glutathione reductase). Beside release of GSSG from the cell, GSSG can also be stored in the cell by the generation of mixed disulfides of GSH and proteins. This process can also be catalyzed by the action of TT (see section Thioltransferase). In this case, GSSG is stored as a glutathione-protein adduct until the redox potential of the cell becomes normal and TT is able to liberate GSSG.

In summary, the amount of GSH in the erythrocyte decreases as a consequence of: (a) conjugation with electrophilic compounds, which is often catalyzed by GST

(b) oxidation of GSH into GSSG by oxidative compounds, (c) peroxidase activity of both GPx and GST and the action of TT with concomitant oxidation of GSH, and (d) the formation of mixed disulfides of GSH and proteins by TT.

Glutathione

GSH is the most important cellular non-protein thiol. To stabilize cysteine in oxidative surroundings, glutamic acid is linked to cysteine by a γ-peptide bond [29]. The reaction is catalyzed by the enzyme γ-glutamylcysteine synthetase [31, 68]. γ-Glutamylcysteine cannot be cleaved by normal intracellular peptidases (specific for an α-peptide linkage; Figure 1.3) [98]. GSH is formed by the linkage of glycine to γ-glutamylcysteine, which is catalyzed by glutathione synthetase. Glycine coupling to the dipeptide counteracts oxidation still further [29].

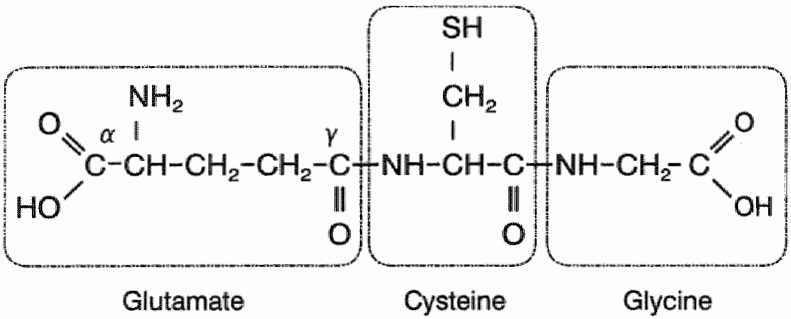

Figure 1.3: glutathione

The erythrocyte has a GT concentration of about 2 mM [31]. For the most part, erythrocyte glutathione is in the reduced state with minor fractions being GSSG. The GT content in erythrocytes is a result of the balance between the rate of synthesis of glutathione and the rate of elimination. Half-life of erythrocyte glutathione is 2–4 days [9, 99]. GSH cannot be transported into the erythrocyte over the erythrocyte membrane [114], but is synthetized in erythrocytes by means of the glutathione synthetizing enzymes γ-glutamylcysteine synthetase and glutathione synthetase [31, 68]. Glutathione synthesis is regulated by the negative feed-back of glutathione on γ-glutamylcysteine synthetase [82], and the availability of the three amino acids [9]. Since erythrocytes lack γ-glutamyltranspeptidase activity [99, 98], transport of amino acids over the erythrocyte membrane may be a problem. Especially the availability of glutamate is considered to be essential since the permeability of the erythrocyte membrane for this amino acid is very low [9]. Export of GSSG or GSH-conjugates over the erythrocyte membrane will be discussed in a separate paragraph on page 18.

Glutathione peroxidase

As a consequence of oxidative stress, membrane lipids may be peroxidized. One of the products generated during lipid peroxidation (LP) is malondialdehyde, which is often used as an indicator for LP [89]. In this thesis the formation of lipid peroxides was determined by means of thiobarbituric reactive substances (TBARS) [93]. Lipid peroxides may be detoxified by glutathione peroxidases. There are two classes of cytosolic glutathione peroxidases, the selenium dependent GPx [32] and the selenium independent GST (see section Glutathione S-transferase). GPx reduces organic hydroperoxides into lipid alcohols. As a consequence GSH is oxidized into GSSG (Figure 1.2) [32]. The enzyme is highly specific for GSH as a reducing substrate and GSH supply is critical for erythrocyte GPx activity [33]. The GPx level in human erythrocytes is usually high and, together with catalase, plays a role in the detoxification of H_2O_2 [32]. GPx activity is sensitive to superoxide anions which can react with the selenolate in the active site of the enzyme [32]. GSSG which is formed during the GPx reaction is reduced by GR at the expense of NADPH. Erythrocytes possess both a hydrophilic and a hydrophobic GPx [32]. As hydrophilic cytoplasmic GPx does not directly reduce hydroperoxo groups in complex lipids [32], cleavage of peroxidized fatty acids by phospholipase must precede the activity of hydrophilic GPx activity [32, 62, 104]. However, (direct) lipophilic GPx activity may be of more importance for the detoxification of lipid membrane hydroperoxides.

Thioltransferase

Erythrocyte TT (EC 1.8.4.1) belongs to the superfamily of thiol-disulfide oxidoreductases. It has a molecular weight of 11300 D and contains four cysteine residues per protein molecule [73, 74]. The average level of activity of TT in erythrocytes (4.6 $U \cdot g^{-1}$ Hb at 25°C) is in the same order of magnitude as GR and G6PDH [29, 35, 36, 70, 73]. TT requires GSH and GR to support its catalytic role in the reduction of oxidized thiol groups in proteins as well as in low molecular mass compounds (see also Figure 1.2). TT along with GR and G6PDH may play a crucial role in erythrocytes sulfhydryl homeostasis by protecting SH-groups on enzymes, hemoglobin and proteins important for membrane integrity. Moreover, at high intracellular GSSG concentrations, TT is also able to store GSSG in the form of mixed disulfides [70].

Glutathione reductase

GR (E.C. 1.6.4.2.; M=125000 D) is a dimer which binds 1 flavin adenine dinucleotide (FAD) per subunit [102]. It has a binding site for GSSG, NADPH and FAD [67, 70, 102]. Electrons are supplied by NADPH and transported via FAD to the active site [67, 70], which is a disulfide [29]. As a result, GSSG is reduced to

GSH [29, 67, 70] (Figure 1.2). As GR is inactivated by NADPH by modification of the reduced enzyme, GR activity is strongly decreased at normal physiological conditions [67]. GSSG protects GR against inactivation and is also able to reactivate the enzyme [67]. In case of oxidative stress, the GSSG concentration increases which activates GR to reduce GSSG into GSH, with concomitant increase of the $NADP^+$ concentration. The increased $NADP^+$ concentration overcomes the inhibition of glucose 6-phosphate dehydrogenase of the hexose monophosphate shunt, so that $NADP^+$ is reduced into NADPH [67], which can be used for the reduction of GSSG. The very low GSSG/GSH ratio is maintained by GR activity [9, 67] and export of GSSG (see further).

Glutathione S-transferase

GST's (E.C. 2.5.1.18) are enzymes which can be found in the cytosol or in microsomes. As erythrocytes do not have organelles, they only possess cytosolic GST. Human cytosolic GST's are divided in class α, μ, π and θ [69, 72]. It depends on the cell type which GST class or classes are expressed [69]. The majority of human erythrocyte GST consists of GST-ρ (90–95 %) which is a GST-π class enzyme [9, 12, 14, 69]. Erythrocytes also contain GST-σ, a minor form of erythrocyte GST, which was characterized and differentiated from the major form by Awasthi *et al.* [6]. More recently, another GST was found which is responsible for the conjugation of methyl halides in erythrocytes [41, 42, 86]. Expression of this θ-class enzyme is known to be polymorfic [41, 42]. Erythrocyte GST has a G (glutathione binding) site and a H (substrate binding) site. The G-site is highly specific for GSH but the H-site has a broad substrate specificity. GSH makes the enzyme catalytically competent by inducing a conformational change as well as providing the functional thiol group for the reaction to be catalyzed [70]. Since the K_m for GST binding in erythrocytes is 0.6 mM [6] and the erythrocyte GSH concentration is about 2 mM, GST will be saturated with GSH.

The nonsubstrate haem can be transported by GST [12]. An interesting quality of GST is its catalytical action upon the conjugation of GSH with electrophiles via substitution or addition of GSH [18], which is usually a detoxification pathway (Figure 1.2). However, a number of compounds can also be activated in this reaction by the formation of episulfonium ions [19]. In vivo, erythrocyte conjugation of electrophiles to GSH may be of great importance as GST-ρ catalyses the conjugation of other electrophiles than e.g. GST-α of the liver [31]. GST-ρ is also able to reduce organic hydroperoxides using GSH as reducing equivalent, with subsequent release of GSSG and an alcohol (Figure 1.2) [12, 22, 29, 75], which is also called the selenium-independent peroxidase activity of GST [56]. The peroxidase activity is restricted to organic hydroperoxides. Unlike glutathione peroxidase (see Glutathione peroxidase), GST is not able to detoxify H_2O_2. The peroxidase activity of GST consists of two reactions, the first one is catalyzed by GST, the second one is a non-enzymatic reaction which requires another GSH molecule [32].

$$ROOH + GSH \xrightarrow{GST} ROSG + H_2O \quad (1.1)$$

$$ROSG + GSH \longrightarrow ROH + GSSG \quad (1.2)$$

As the organic hydroperoxide must be released from the cellular membrane before it can be reduced by cytosolic GST, the action of phospholipase is a requirement [56,57,62,104]. This was also the case in GPx-mediated reduction of organic hydroperoxides.

Export of glutathione and glutathione conjugates

Both GSSG and conjugates of GSH and electrophiles (glutathione S-conjugates) can be removed from the erythrocyte by means of active transport [9]. GSSG can be exported by two different ATP-ases, one with a high affinity for GSSG (K_m=0.1 mM) and one with a low GSSG affinity (K_m=7.1 mM) [9]. At low intracellular GSSG concentrations, GSSG will mainly be transported by the high affinity carrier. However, its capacity is limited as the V_{max} is 20 nmoles·ml^{-1}h^{-1} [61]. The V_{max} of the low affinity carrier is ten times higher, so that GSSG export by this carrier may be important in case of oxidative stress. The low affinity carrier is also able to transport the S-(2,4 dinitrophenyl)-glutathione. The K_m values for the glutathione S-conjugate and ATP are 0.29 mM and 1.0 mM respectively [7,64]. Awasthi *et al.* demonstrated that the transport of glutathione S-conjugates is probably the most important function of the low affinity carrier *in vivo*, since GSSG was not transported anymore in the presence of S-(2,4 dinitrophenyl)-glutathione even though the transport of S-(2,4 dinitrophenyl)-glutathione was not inhibited by GSSG concentrations up to 11.8 mM [7]. This means that glutathione S-conjugates limit the export of GSSG by the low affinity carrier. Since the high affinity carrier was not affected by the glutathione S-conjugate, it is likely that this carrier serves to protect the erythrocyte from the toxic intracellular effects of GSSG [9]. Since S-(2,4 dinitrophenyl)-glutathione also inhibits GR activity [92], glutathione S-conjugates may have an unfavorable effect on the GSH/GSSG redox status of the cell, firstly by the consumption of GSH required for conjugate formation, secondly by the inhibition of GSSG transport from the cell by the conjugate, and thirdly by inhibition of GR. Glutathione S-conjugates may also be able to affect GST activity, which was reported for e.g. S-(2-chloro 4-nitrophenyl)-glutathione [92].

The rat liver

As discussed above, xenobiotics may affect biochemical reactions and targets in erythrocytes. Various organs can transform a non-reactive parent compound

into metabolites that may affect erythrocytes. As the liver is the organ with the highest metabolic capacity, the effect of the addition of rat liver metabolizing systems to *in vitro* incubations with erythrocytes is investigated in this thesis. For this reason, several biochemical aspects of the rat liver will be discussed.

The rat liver plays an important role in the metabolism of carbohydrates, fat and proteins, and in the biotransformation of xenobiotics. It is mainly composed of hepatocytes (60–65 % of the total cell number [37] and 78 % of the liver volume [13]), which are responsible for a large fraction of the metabolic activity of the liver. A cell layer of hepatocytes is arranged along the sinusoids that spans the portovenous distance. Afferent vessels are the hepatic arterioles and portal venules and efferent vessels are the central vein and the bile ducts [40]. The high oxygen consumption of the liver leads to a steep gradient along the sinusoids as well as from the center of the sinusoid to the plasma membranes of the cells and intracellularly to the mitochondria [37]. Structural and functional differences between rat hepatocytes from different locations have been observed, especially regarding enzyme activities (see below) [49].

Biotransformation of xenobiotics by the liver can be divided in phase I and phase II activity. Phase I activity comprises oxidation, reduction and hydrolysis of xenobiotics and phase II activity includes conjugation or synthetic reactions [3, 60]. Although phase I and II metabolism are regarded as detoxifying reactions, some chemicals may also be converted into more toxic metabolites [39].

Phase I reactions add functional groups (e.g. –OH, –SH, $–NH_2$, –COOH) to the parent compound. Enzymes of the cytochrome P-450 gene superfamily are considered the most important phase I enzymes [60]. Cytochrome P-450 catalyzes the mono-oxygenation of a substrate in the presence of oxygen and reducing equivalents [105]. In man, minor changes in the oxygen supply to the liver may depress the cytochrome P-450 activity as the K_{mO_2} of cytochrome P-450 (10–15 μM) is in the same range as the mean *in vivo* intra-hepatic oxygen concentration (about 35 μM) [3]. The cytochrome P-450 enzymes are embedded in the smooth endoplasmic reticulum [105], and as such are a constituent of microsomal preparations. The total cellular cytochrome P-450 content increases from the periportal to the perivenous site of the liver lobule [38, 47, 88, 95, 100, 112]. Since the oxygen concentration decreases in that direction, the O_2 supply for cytochrome P-450 is even more critical than would be expected from the mean intrahepatic O_2 concentration. Treatment of animals with phenobarbitone leads to an increase in the perivenous cytochrome P-450 content and to an increase in the proliferation of the endoplasmic reticulum [46, 47].

By means of phase II metabolism, functional groups on a xenobiotic may be coupled to endogenous substrates. The functional groups may be produced by phase I metabolism, but there are also compounds that can immediately undergo conjugation reactions [3]. In this way, reactive compounds may be detoxified in

the hepatocyte so that other cells (including erythrocytes) will not be exposed to these reactive compounds, as would be the case if they would be able to leave the hepatocyte unchanged. A great number of phase II reactions are known to occur in the liver, but only the reactions that are of importance for the understanding of the material presented in this thesis will be described.

- Glucuronidation is one of the most important phase II reactions. In this reaction, uridine diphosphate glucuronic acid (UDP-glucuronic acid) is coupled to the functional group on the acceptor molecule. The reaction is catalyzed by UDP-glucuronosyl transferase which has a very broad substrate specificity [60]. UDP-glucuronosyltransferase activity is higher in perivenous hepatocytes [3, 24]. Glucuronide conjugates may be deconjugated by the hydrolase β-glucuronidase, which seems to be evenly distributed across the liver lobule [24, 50].
- Sulfation is an important conjugation reaction for hydroxyl groups. The reaction is catalyzed by sulfotransferases, which transfer activated sulfate to the hydroxyl group present on phenols and aliphatic alcohols [60]. Sulfotransferase activity was higher in periportal than in perivenous hepatocytes when umbelliferone was tested [3, 24]. Aryl sulfatases may degrade aryl-sulfate conjugates of xenobiotics. They seem to be evenly distributed over the liver lobule [24, 50].
- Glutathione S-transferases catalyze the conjugation of electrophiles with the nucleophilic sulfhydryl of GSH [18]. The hepatic GT concentration is depleted in this way. Hepatocytes possess both cytosolic GST's and GST's associated with the endoplasmic reticulum [75]. There is a large structural difference between the two types of GST's as cytosolic GST is a dimer [57, 69] and endoplasmic reticulum bound GST has three subunits [75]. The main cytosolic GST class found in hepatocytes is GST-α [69]. Hepatic cytosolic GST activity which is responsible for conjugation of GSH to electrophiles, is found predominantly in isolated perivenous rat hepatocytes [54, 55].

Partly due to the action of GST, hepatocyte GSH can be depleted. Beside GSH conjugation, the hepatic glutathione pool can also be depleted by transport of GSSG over the hepatocyte membrane. This occurs when the reductive capacity of the hepatocyte is not sufficient to maintain the low GSSG/GSH ratio [1]. GSSG is formed in the peroxidase reaction of GST in which peroxidized lipids are reduced into alcohols [32]. Unlike the transferase activity, the peroxidase activity of GST was found to be higher in the periportal area [54]. This may be explained by the distribution of GST isoenzymes over the lobulus. GPx is able to reduce both H_2O_2 and peroxidized lipids with concomitant GSSG formation [32]. In accordance with the peroxidase activity of GST, GPx activity was also found to be higher in periportal hepatocytes [54]. GSH is also used as reducing equivalent by thioltransferase in the reduction of protein disulfides or mixed disulfides of glutathione and protein, though its activity in rat liver is low [35]. Beside conjugation

of glutathione with electrophiles, cytosolic GST's are involved in the formation of Δ^5 3-ketosteroids and prostaglandines [12]. They also bind the nonsubstrates bilirubin and other bile acids [12].

The synthesis of glutathione by the liver is 0.5 nmoles·mg protein^{-1}·min^{-1} [89], and is normally higher than the glutathione consumption. Under normal conditions liver GSH is released into plasma and bile and used by other organs. Neither GSH nor GSSG is taken up by the liver [1], as hepatocytes do not have γ-glutamyltranspeptidase activity [37,59]. According to Kera *et al.*, Shimizu and Morita, Smith *et al.* and Väänäen [53,90,94,111] periportal hepatocytes contain more glutathione. This in contrast with Asghar *et al.*, Deml and Oesterle, Schön and Speisky *et al.* [5,20,85,97], who found a more evenly distributed glutathione concentration across the lobule. Intracellularly, glutathione is found both in the cytosol (90 % of the GT concentration) and in mitochondria (10 % of the GT concentration) [81,83,110]. Mitochondria lack the ability to synthetize GSH [83] and are unable to export GSSG [110]. Depletion of GSH by electrophiles or oxidation into GSSG by oxidative compounds is compatible with life as long as the mitochondrial GSH pool is not depleted too much. Both cytosolic GSSG and GSH adducts (e.g. S-(2,4 dinitrophenyl)-glutathione) are exported from the hepatocytes into bile [1,48,91,92]. There is even a mutual competition between transport of GSSG and GSH-conjugates [91,92]. In this way, GSH conjugates may have an unfavorable effect on the GSH/GSSG redox status in the cell, firstly by the consumption of GSH required for conjugate formation, and secondly by the inhibition of GSSG transport from the cell by the conjugate which was also described for erythrocytes [91,92].

Objectives and outline of the thesis

Exposure of living organisms to high doses of xenobiotics may lead to adverse effects. Chronic exposure of living organisms to low doses of xenobiotics may also lead to impairment of health [26]. At an earlier stage in time, biological parameters at the cellular level may be affected [26]. In the preceding text it was demonstrated that human erythrocytes possess an interesting amount of targets and biochemical reactions to study the effect of xenobiotics. An other advantage of human erythrocytes is that they are readily available.

Since *in vivo* there is a close contact between blood and hepatocytes, non-reactive xenobiotic compounds may be metabolised by the liver into compounds that cause biological effects in erythrocytes. In this thesis main attention is directed to the interplay between erythrocytes and hepatocytes with special emphasis to the bioactivation and inactivation capacity of liver and blood. To enable such studies, several *in vitro* techniques combining erythrocytes and rat liver metabolizing systems had to be developed and evaluated, as is illustrated in Figure 1.4.

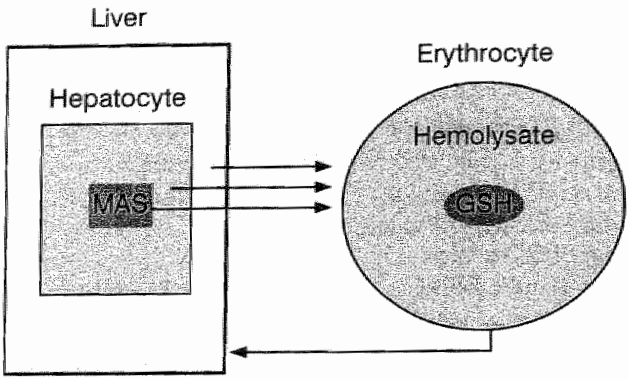

Figure 1.4: Diagram that outlines the incubations performed in this thesis. Glutathione solution (GSH), hemolysate and erythrocytes were used as GSH-containing target systems. As compounds may have to be metabolised to deplete GSH, metabolically activating systems were also added to the incubations. The metabolically activating systems used in this thesis consisted of microsomal activating system (MAS), hepatocytes and total liver. Finally, the ability of erythrocytes to affect the viability and biotransformational capacity of the total liver was evaluated (↩).

Several compounds with known effects were used as positive controls to develop and understand the test systems. Compounds tested in this thesis can be divided into (i) compounds with known electrophilic capacity (iodoacetamide, N-ethylmaleimide and diethyl maleate), (ii) a strong oxidator (hydroxylamine) and (iii) compounds that have to be metabolised to be able to deplete GT (cyclophosphamide, 3-hydroxyacetanilide, 2-methylfurane). Subsequently, the test systems were applied to several test compounds with unknown effects. Two pesticides, monuron and monulinuron, were tested since their metabolite 4-chloroaniline is able to cause hemoglobin adducts in rats [84]. Because the adducts are attached to the thiol-group of hemoglobin, other thiols may also be affected. The industrial chemicals cyclohexanone oxime, acetaldoxime and methyl-ethyl ketoxime were tested since oximes are known hematotoxic compounds in rats. Among others, cyclohexanone oxime causes hemoglobin oxidation and an increase in circulating reticulocytes [21, 34] and methyl-ethyl ketoxime causes a decrease in erythrocyte count and hemoglobin content [63]. Workers exposed to dimethylacetamide (DMAc) are able to excrete the metabolite monomethylacetamide [71, 15, 52]. The metabolism of DMAc is believed to be confined to the liver [51]. In our studies DMAc is used to test whether an isolated rat liver is able to metabolize this compound. Furthermore, DMAc is also used to test whether erythrocytes affect DMAc elimination kinetics in an isolated perfused rat liver.

The outline of the thesis is as follows:

- *Microsomal* preparations were used in **chapter 2** as metabolic activating system. GT depletion by active compounds was studied as parameter of toxicity.
- The metabolic capacity of *microsomal* activating system and intact *hepatocytes* was compared in **chapter 3**. Depletion of GT or GSH was used as toxicological parameter.
- In **chapter 4** the biotransformational capacity of an *isolated total liver* was studied by means of the investigation of the elimination kinetics of DMAc. Thereupon **chapter 5** describes the influence on DMAc elimination kinetics by addition of erythrocytes to the liver perfusion system.
- **Chapter 6** deals with a comparative study. It describes whether the different combinations with either *microsomal preparations* or *hepatocytes* or *isolated total liver* and erythrocytes are useful to establish the toxicological profile of hydroxylamine and several oximes. Beside GT, several other endpoints have been measured.
- In **chapter 7** the results in the studies performed in this thesis are discussed, and they are summarised in **chapter 8**.

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Chapter 2

Glutathione depletion in human erythrocytes as an indicator for microsomal activation of cyclophosphamide and 3-hydroxyacetanilide

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Summary

A model system for the detection of reactive metabolites, using glutathione depletion after microsomal activation, has been described previously. We developed a battery of complementary test systems using rat liver microsomes for metabolism and aqueous glutathione solutions, human erythrocytes or hemolysate derived there from, as target. Reactive metabolite formation and the ability of metabolites to pass the erythrocyte membrane were tested using 3-hydroxyacetanilide (3-HAA) and cyclophosphamide (CP) as substrates. Neither unchanged 3-HAA nor CP depleted glutathione in erythrocytes or in aqueous reduced glutathione solutions (GSH solutions). Addition of normal rat liver microsomes or liver microsomes from rats pretreated with phenobarbital (PB microsomes) induced a 3-HAA/CP concentration-dependent glutathione depletion in both systems. With PB microsomes, higher depletions were found. While unchanged 3-HAA did not deplete aqueous GSH solutions or glutathione in erythrocytes, a significant depletion in hemolysate was found. The results indicate that both CP and 3-HAA metabolites are able to pass through the erythrocyte membrane. While both substances can metabolically be activated by rat liver microsomes, only 3-HAA can be activated by soluble factors in erythrocytes. However, unchanged 3-HAA has no effect on GSH in erythrocytes. This might be caused by an inability of unchanged 3-HAA to enter the erythrocyte. More generally, an adequate combination of the test systems described can be used to detect (a) the reactivity of unchanged substances and their metabolites, and (b) the ability of unchanged substances and their reactive metabolites to pass through the erythrocyte membrane.

Introduction

The tripeptide glutathione acts as an efficient scavenger of reactive electrophilic substances in conjunction with glutathione-dependent enzymes [6, 25, 26, 31]. The glutathione thiol has a higher affinity for reactive (electrophilic) compounds than protein thiols [8] or nucleophilic centers in DNA [5] and it also protects protein thiols against oxidation [6, 31]. Depletion of intracellular glutathione will have consequences for these protective functions.

The liver is susceptible to glutathione depletion, as this organ has a very high phase I metabolic activity, often leading to the formation of reactive metabolites [17]. Hepatocytes have a high glutathione concentration (10–12 mM)[22]. Next to the liver, blood also contributes to glutathione-dependent metabolism. The role of blood is important because of its volume, the glutathione concentration (2 mM) in the erythrocytes and its presence all over the body. The glutathione concentration in human erythrocytes is much higher than in plasma [26].

The contribution of erythrocytes to the protection against reactive metabolites depends on the ability of metabolites to pass the erythrocyte membrane [10].

The aim of the present study was to develop a system able to discriminate between:

1. substances which react with GSH without preceding metabolic activation and substances which need metabolic activation; and
2. substances or reactive metabolites able and unable to pass the erythrocyte membrane.

To study these objectives several glutathione sources were used, i.e., aqueous reduced glutathione solutions (GSH solutions), human erythrocytes and hemolysate derived there from, incubations with and without rat liver microsomes (normal or phenobarbital pretreated rat liver microsomes) were compared and 3-HAA and CP, two test compounds which are known to deplete GSH only after metabolic activation [13], were used to test our model.

The sum of both reduced and oxidized glutathione was determined (= total glutathione (GT)). The loss of GT (GT depletion) as a consequence of the incubation of 3-HAA or CP with a metabolizing system was used as a measure for reactive metabolite formation.

Materials and Methods

Chemicals

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), glutathione reductase (EC 1.6.4.2), Glucose-6-phosphate (G6P), NADP, cyclophosphamide (CP; CAS: 6055-19-2) and 3-hydroxyacetanilide (3-HAA; CAS: 621-42-1) were obtained from Sigma (St. Louis, MO, USA). Water was demineralized and microfiltrated before use. All other chemicals were of analytical grade quality.

Animals

Male Brown Norway rats (BN/M) of about 250 grams were used. A non-albino strain was chosen as biotransformation of xenobiotics is lower in albino strains [29]. Housing was as previously described [27]. When liver microsomes from rats pretreated with phenobarbital (PB microsomes) were used, phenobarbital was added to the drinking water (1 $g \cdot l^{-1}$) during one week prior to the preparation of microsomes.

Preparation of microsomal activating system

Animals were anesthetized using pentobarbital (Narcovet^{TM}; 0.2 ml/100 g rat). Livers were removed and homogenized in three volumes of ice-cold phosphate buffer (50 mM and pH 7.4), using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged at 9000×g for 20 minutes at 4°C. The microsomal fraction was sedimented from the supernatant by 40 minutes centrifugation at 110000×g at 4°C. The pellet was resuspended in the same ice-cold buffer and diluted so that the microsomal fraction of 3 g liver was suspended in 1 ml buffer. Microsomal activating system was composed of microsomes (final protein concentration 1 $mg \cdot ml^{-1}$) and NADPH regenerating system (0.5 mM NADP, 5.0 mM $MgCl_2$, 5.0 mM G6P and 1.0 $U \cdot ml^{-1}$ G6PDH)

Preparation of erythrocytes and lysate

Human blood was obtained from the Red Cross Blood Bank "Zuid Limburg", collected in sterile vacuum EDTA vessels and kept overnight at 4°C. Erythrocytes were pooled, washed three times using saline and diluted three times in 100 mM Na_2HPO_4/KH_2PO_4 buffer (final hemoglobin concentration about 100 $g \cdot l^{-1}$). Lysate was prepared from packed erythrocytes by dilution with ice-cold water (1:2). The cellular debris was removed by centrifugation (5 minutes, 2000×g).

Incubations

Human erythrocytes (final Hb concentration 23 $g \cdot l^{-1}$) or aqueous GSH solutions (final concentration 450 μM) were suspended in 100 mM Na_2HPO_4/KH_2PO_4 buffer (final volume 3 ml). Incubations were performed both with and without microsomal activating system. Microsomes of both normal and phenobarbital pretreated animals were tested. The complete test system without test substance was preincubated for 5 minutes at 37°C in a shaking waterbath (80 rev./min). Thereafter, 1 ml 3-HAA or CP (final concentration between 0 and 2 mM) was added and incubated for 1 hour. All experiments were performed three times on different days and with different blood samples. Each day, all incubations were performed in triplicate. Separate experiments were performed for test compound concentrations between 0 and 0.5 mM and between 0.5 and 2.0 mM.

To verify metabolic activity of the erythrocytes themselves, GT depletion in hemolysate was also tested. After preincubating hemolysate for 5 minutes at 37°C in a shaking waterbath (80 rev./min) 1 ml 3-HAA or CP (final concentrations 0, 0.5 1.0 and 2.0 mM) was added and incubated for 1 hour. After 1 hour incubation, the reaction was stopped by the addition of an equal volume of 5 % (w/v) trichloroacetic acid (TCA). Erythrocytes were packed before the addition of TCA by centrifugation (5 minutes, 2000×g). The degree of concentration was

determined by measuring hemoglobin concentrations of the test incubate before and after concentration. The amount of erythrocytes pipetted for determination of the hemoglobin concentration was weighed.

Analytical procedures

GT in the TCA supernatants was determined after dilution (9-fold) with buffer by the cyclic oxidation reduction method described by Anderson [2]. Microsomal protein content was determined according to Lowry *et al.* [24]. Hemoglobin concentrations were determined by measuring cyanmethemoglobin according to van Kampen and Zijlstra [35].

Statistical analysis

GT depletion was analyzed by linear regression using a model including day of experiment, concentration, metabolizing system and concentration times metabolizing system as explanatory variables. Using that model, we tested whether slopes of GT depletion versus concentration were significant for each of the metabolizing systems and whether these slopes were different from each other, correcting for the day of the experiment.

Results

3-Hydroxyacetanilide

Depletion of GT by addition of 3-HAA with and without microsomal activation is shown in Figures 2.1 and 2.2 for aqueous GSH solutions and erythrocytes, respectively. Without metabolic activating system, GT was not depleted in both systems. Microsomal activation of 3-HAA resulted in GT depletion, both in incubations with aqueous GSH solutions and in erythrocytes. This depletion was stronger when PB microsomes were used.

For aqueous GSH solutions, the depletion increased from 14 (SD=23) nmoles·ml^{-1}h^{-1} at 0.1 mM 3-HAA to 70 (SD=38) nmoles·ml^{-1}h^{-1} at 2.0 mM ($p = 0.0001$) with normal microsomes and from 67 (SD=30) nmoles·ml^{-1}h^{-1} at 0.1 mM 3-HAA to 91 (SD=16) nmoles·ml^{-1}h^{-1} at 2.0 mM with PB microsomes ($p = 0.0001$). GT depletion in incubations with aqueous GSH solutions and PB microsomes was maximal at a 3-HAA concentration of about 0.3 mM (99 nmoles·ml^{-1}h^{-1}, SD=27). For erythrocytes, GT depletion was also statistically significant and was 24 (SD=1) and 61 (SD=3) nmoles·ml^{-1}h^{-1} at 2 mM 3-HAA with normal ($p = 0.0040$) and PB microsomes ($p = 0.0001$), respectively.

GT depletions using normal microsomes were significantly higher compared to incubations without microsomes for both aqueous GSH solutions ($p = 0.0004$) and

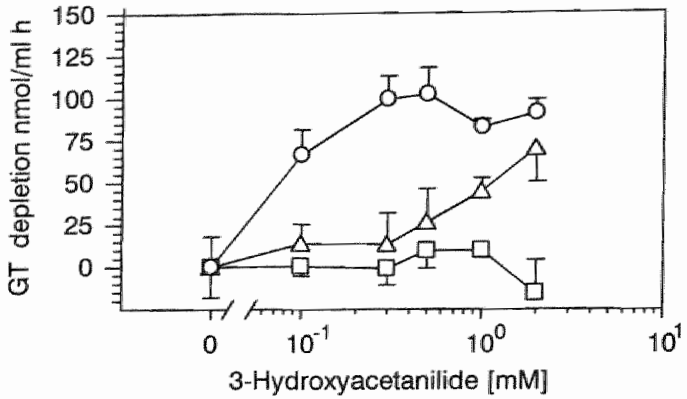

Figure 2.1: GT depletions in incubations of aqueous GSH solutions with 3-HAA. Microsomes of PB pretreated (○) and untreated (△) rats were used. Controls (□) were incubated without microsomes. The means of GT depletions and half the SD of GT concentrations are shown. The number of experiments was n=6 for 0 and 0.5 mM 3-HAA and n=3 for the other concentrations tested. GT depletion was significantly related to the 3-HAA concentration, for both normal ($p = 0.0001$) and PB microsomes ($p = 0.0001$). Compared to incubations without microsomes, GT depletions were significantly higher when normal ($p = 0.0004$) or PB ($p = 0.0002$) microsomes were used.

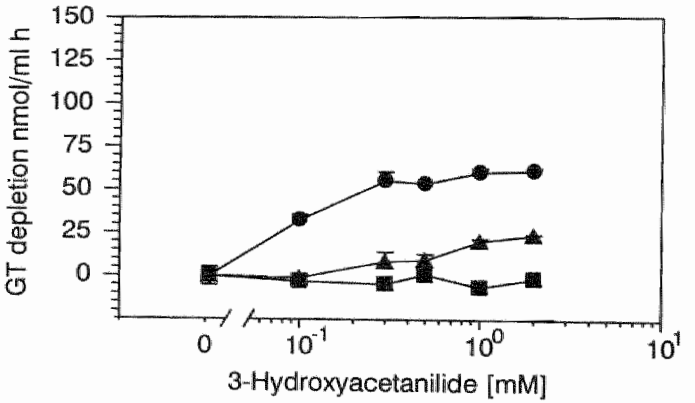

Figure 2.2: GT depletions in incubations of human erythrocytes with 3-HAA. Microsomes of PB pretreated (●) and untreated (▲) rats were used. Controls (■) were incubated without microsomes. The means of GT depletions and half the SD of GT concentrations are shown. The number of experiments was n=5 for 0 and 0.5 mM 3-HAA and n=3 for the other concentrations tested. GT depletion was significantly related to the 3-HAA concentration, for both normal ($p = 0.0040$) and PB microsomes ($p = 0.0001$). Compared to incubations without microsomes, GT depletions were significantly higher when normal ($p = 0.0329$) or PB ($p = 0.0002$) microsomes were used.

erythrocytes ($p = 0.0329$). At 3-HAA concentrations between 0 and 0.5 mM, GT depletions in incubations with PB microsomes were higher than GT depletions in incubations with normal microsomes for both aqueous GSH solutions ($p = 0.0001$) and erythrocytes ($p = 0.0001$); above 0.5 mM saturation of 3-HAA metabolism is reached in PB microsomes.

A concentration-dependent GT depletion could be observed when lysate was incubated with 3-HAA ($p = 0.0018$). Absolute depletions were 12 (SD=22), 25 (SD=19) and 36 (SD=6) nmoles·ml^{-1}h^{-1} with 0.5, 1.0 and 2.0 mM 3-HAA, respectively.

Cyclophosphamide

Figures 2.3 and 2.4 show depletion of GT by addition of CP with and without microsomal activation for aqueous GSH solutions and erythrocytes, respectively. In the absence of microsomal activation, CP induced no GT depletion in incubations in which aqueous GSH solutions or erythrocytes were used. Microsomal activation of CP resulted in GT depletion both in incubations with aqueous GSH solutions and in erythrocytes. GT depletion was higher when PB microsomes were used. GT was not depleted when lysate was incubated with CP.

GT depletion in incubations with aqueous GSH solutions increased from 16 (SD=23) nmoles·ml^{-1}h^{-1} at 0.5 mM CP to 105 (SD=8) nmoles·ml^{-1}h^{-1} at 2 mM ($p = 0.0001$) with normal microsomes and from 93 (SD=44) nmoles·ml^{-1}h^{-1} at 0.5 mM CP to 218 (SD=30) nmoles·ml^{-1}h^{-1} at 2.0 mM with PB microsomes ($p = 0.0001$). For erythrocytes, GT depletions increased from 35 (SD=11) nmoles·ml^{-1}h^{-1} at 0.5 mM CP to 92 (SD=6) at 2 mM ($p = 0.0001$) with normal microsomes and from 96 (SD=12) nmoles·ml^{-1}h^{-1} at 0.5 mM CP to 90 (SD=13) nmoles·ml^{-1}h^{-1} at 2 mM ($p = 0.0001$) with PB microsomes. In incubations with erythrocytes, all glutathione was consumed at 0.5 mM CP when PB microsomes were tested. In incubations with normal microsomes, GT depletion in incubations with erythrocytes was comparable with incubations with aqueous GSH solutions.

GT depletions in incubations with normal microsomes were significantly higher compared to incubations without microsomes for erythrocytes ($p = 0.0001$) and aqueous GSH solutions ($p = 0.0017$), respectively. At CP concentrations between 0 and 0.5 mM, GT depletions in incubations with PB microsomes were higher than GT depletions in incubations with normal microsomes for both aqueous GSH solutions ($p = 0.0001$) and erythrocytes ($p = 0.0001$), above 0.5 mM saturation of CP metabolism is reached in PB microsomes.

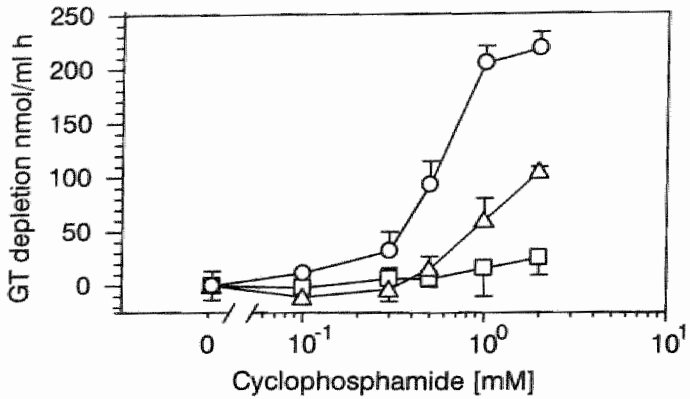

Figure 2.3: GT depletions in incubations of aqueous GSH solutions with CP. Microsomes of PB pretreated (○) and untreated (△) rats were used. Controls (□) were incubated without microsomes. The means of GT depletions and half the SD of GT concentrations are shown. The number of experiments was n=5 for 0 and 0.5 mM 3-HAA and n=3 for the other concentrations tested. GT depletion was significantly related to the 3-HAA concentration, for both normal ($p = 0.0001$) and PB microsomes ($p = 0.0001$). Compared to incubations without microsomes, GT depletions were significantly higher when normal ($p = 0.0017$) or PB ($p = 0.0001$) microsomes were used.

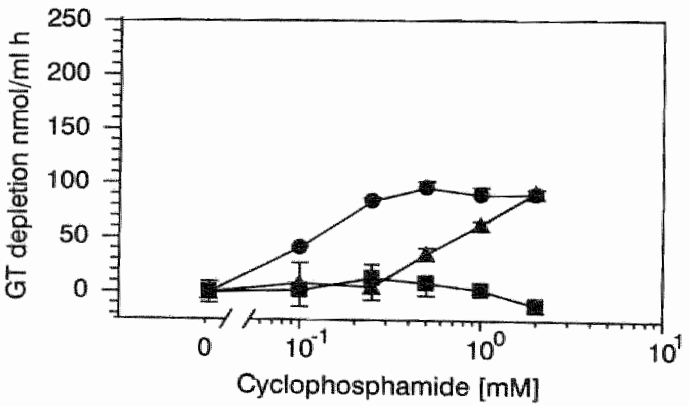

Figure 2.4: GT depletions in incubations of human erythrocytes with CP. Microsomes of PB pretreated (●) and untreated (▲) rats were used. Controls (■) were incubated without microsomes. The means of GT depletions and half the SD of GT concentrations are shown. The number of experiments was n=6 for 0 and 0.5 mM 3-HAA and n=3 for the other concentrations tested. GT depletion was significantly related to the 3-HAA concentration, for both normal ($p = 0.0001$) and PB microsomes ($p = 0.0001$). Compared to incubations without microsomes, GT depletions were significantly higher when normal ($p = 0.0001$) or PB ($p = 0.0001$) microsomes were used.

Discussion

GSH plays a key role in the detoxification of electrophiles. It also reduces protein and hemoglobin thiols and prevents the formation of hemoglobin and DNA adducts [8]. GSH depletion has been used previously to quantify the formation of reactive metabolites in an *in vitro* test system containing aqueous GSH solutions and microsomes [13, 15, 14]. We used a similar system for detection of reactive metabolites formed from 3-HAA and CP. In addition, the ability of the metabolites formed to enter a target cell was evaluated. For this purpose GT depletion in human erythrocytes added to the microsomal system was determined. Finally, the metabolic activity of the erythrocytes themselves, which contain both oxygenase [7,32] and glutathione S-transferase activity [9], was tested using hemolysate.

In this study 3-hydroxyacetanilide (3-HAA), an analogue of paracetamol with antipyretic and analgetic properties [33] and cyclophosphamide (CP), which is used as a chemotherapeutic agent and as an immunosuppressor [28], were tested. The liver is the principal site for biotransformation of CP [11,34] and 3-HAA [33]. CP is metabolised by hepatic microsomal enzymes to 4-hydroxycyclophosphamide. This initial transformation is rate limiting[19]. From 4-hydroxycyclophosphamide, equimolar amounts of phosphoramide mustard and acrolein are produced [20]. Acrolein forms a conjugate with GSH and is excreted into the urine as 3-hydroxypropylmercapturic acid. The rate of biotransformation of CP increased 2–3-fold in patients treated with phenobarbital [4,21] and it was concluded that the metabolism of CP is mediated by liver mixed-function oxidases. Pretreatment of rats with SKF 252A [34] and cimetidine [3] inhibited CP metabolism, also indicating that CP metabolism is mediated by cytochrome P-450. 3-HAA is metabolised by mouse hepatic microsomes to 2',5'-dihydroxyacetanilide (DHAA; note that the 3'-OH group is renumbered to 5' in this structure), 3',4'-DHAA and 2',3'-DHAA. The 2',5'-DHAA metabolite is oxidized further to an electrophilic semiquinone and/or quinone species [33].

GSH plays a significant role in protection against CP and 3-HAA toxicity and covalent binding. CP toxicity in rats pretreated with L-buthionine-*SR*-sulfoximine which inhibits GSH synthesis, was enhanced [12]. CP caused a dose-dependent reduction in pulmonary GT stores in rats [1]. Covalent binding to macromolecules after CP treatment in mice was enhanced by diethylmaleate (a GSH depletor) and blocked by cysteine [18], indicating that GSH plays a protective role against covalent binding of CP metabolites. *In vitro*, a substantial depletion of cellular GSH was accompanied by a significant reduction of tumor cell reproductive capacity [23]. *In vivo* administration of 3-HAA to hamsters caused a significant decrease in hepatic GT only at doses of 550 $mg \cdot kg^{-1}$ and higher [30]. Metabolites of 3-HAA induce covalent binding to hepatic protein [30]. Cysteine and GSH were found to inhibit this binding [33].

After oxidation, erythrocyte glutathione is either excreted by erythrocytes [6]

or bound to proteins (mixed disulfides) [16]. Excretion of oxidized glutathione or formation of mixed disulfides in erythrocytes would be identified as depletion of total GT. Concentrations of GT in the supernatants were determined. However, no GT could be detected in these preparations, suggesting the formation of protein-glutathione mixed disulfides or glutathione-metabolite complexes.

GT was not depleted when 3-HAA or CP were incubated with aqueous GSH solutions or erythrocytes. GT was depleted in both systems when 3-HAA or CP were tested after the addition of microsomal activating system. This indicates not only that metabolic activation is necessary before glutathione depletion can occur, but also that such an activation does not occur within the erythrocyte or that the unchanged substances are not able to enter the erythrocyte. GT concentrations in hemolysates decreased when 3-HAA was used as a test substrate. This indicates that erythrocytes contain soluble factors able to activate 3-HAA. Therefore, it is likely that 3-HAA itself is not able to enter the erythrocyte.

The addition of normal microsomes with a NADPH regenerating system to incubations with aqueous GSH solutions or erythrocytes caused GT depletions which were concentration dependent for both test compounds. Combined with the results found without added microsomes, this proves that metabolites of both 3-HAA and CP are able to pass the erythrocyte membrane and to deplete GT. GT depletions were higher for both 3-HAA and CP when PB microsomes were tested. As CP and 3-HAA are mainly metabolised by hepatic microsomal enzymes [19,33], these results are in agreement with the literature.

GSH depletions in incubations with aqueous GSH solutions and normal or PB microsomes and 1 mM 3-HAA of 39 nmoles·ml^{-1}30 min^{-1} and 209 nmoles·ml^{-1}30 min^{-1}, respectively have been reported [13]. As only 200 μM aqueous GSH solution was added to their incubations, GSH was totally depleted using PB animals. We found GT depletions of 45 (SD=16) nmoles·ml^{-1}h^{-1} and 83 (SD=9) nmoles·ml^{-1}h^{-1} for normal and PB microsomes, respectively, under the same conditions. The lower GT depletion velocity found for PB microsomes in the present study might be caused by the different rat strain used or differences in phenobarbital treatment regimes. GT depletions in incubations with CP (1 mM) are higher in comparison with those of Fry for both normal and PB microsomes. Fry reported GSH depletions of 15 and 85 nmoles·ml^{-1}30 min^{-1} for normal and PB microsomes, respectively. At CP concentrations of 1.0 mM, we found GT depletions of 60 (SD=40) and 205 (SD=31) nmoles·ml^{-1}h^{-1} respectively. This also may be a result of differences in rat strain or pretreatment conditions. The differences in relative glutathione depletions in the two studies for CP (higher in our study) and 3-HAA (lower in our study) might indicate that CP and 3-HAA are metabolised by different P-450 isoenzymes.

In metabolically activated incubations in which CP was used as a test substrate, GT depletions in incubations with aqueous GSH solutions were comparable with

incubations with erythrocytes. In contrast, GT depletions in incubations with 3-HAA were about two times lower in incubations with erythrocytes. These results indicate that the relative intracellular concentration of reactive metabolite concentration (compared to extracellular concentrations) is higher for CP than for 3-HAA. This may be caused by a difference in uptake or in intracellular metabolism.

Based on our results, it may be concluded that while unchanged 3-HAA and CP do not deplete GT, microsomal activation products are able to enter the erythrocyte and to deplete GT. An appropriate combination of test systems as described here can be used to discriminate direct reactive substances from those which have to be metabolised, while also metabolites able to pass the (erythrocyte) cell membrane can be discriminated from others.

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Chapter 3

Glutathione depletion in human erythrocytes and rat liver; a study on the interplay between bio-activation and inactivation functions of liver and blood

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Summary

The interplay between bioactivation and inactivation functions of human erythrocytes and rat liver was studied. Glutathione depletion was used as a measure for the amount of GSH-reactive compound. The direct electrophiles iodoacetamide (IAcA), N-ethylmaleimide (NEM) and diethyl maleate (DEM), were able to deplete reduced glutathione (GSH) in both incubations with aqueous GSH solution and erythrocytes. This indicates that these compounds can pass the erythrocyte membrane. Cyclophosphamide (CP), 3-hydroxyacetanilide (3-HAA) and 2-methylfurane (2-MF) needed metabolic activation by rat liver microsomes to deplete glutathione in incubations with aqueous GSH solution or erythrocytes. In these incubations the sum of both reduced and oxidized glutathione (=total glutathione (GT)) was measured. It indicates that GSH-reactive metabolites are generated out of CP, 3-HAA and 2-MF by microsomes and that these metabolites can pass the erythrocyte membrane. As GT depletions were higher when microsomes of phenobarbital pretreated rats were tested, the metabolites were (also) generated by phenobarbital inducable enzymes. GT was also depleted in incubations with hemolysate and 3-HAA or 2-MF, but not in incubations with aqueous GSH solution. This indicates that erythrocyte cytosol can metabolize 3-HAA and 2-MF into GSH-reactive compounds. The pesticides monuron and monulinuron did not affect GT concentrations when aqueous GSH solution, hemolysate or erythrocytes with or without microsomal activating system were tested. When hepatocytes were incubated with CP or 3-HAA (2 mM), the hepatic GT concentration was depleted (about 1.5 mM in both cases). The hepatocytes were able to excrete GSH-reactive metabolites generated from CP or 3-HAA (21 % and 77 % resp.). GT was not depleted in co-incubations of hepatocytes and erythrocytes with 3-HAA, which may be explained by uptake limitation of the GSH-reactive metabolites into the erythrocytes.

Introduction

Reduced glutathione (GSH) and glutathione S-transferase (GST) play a key role in the *in vivo* detoxification of electrophiles [6, 21, 22, 27]. As human erythrocytes contain a great amount of GSH (about 2 mM) [10] and a relatively high GST activity (1–6 $U \cdot g^{-1}$ hemoglobin) [33], they may play an important role in the detoxification of electrophiles. Various electrophiles are known to cause glutathione depletion in erythrocytes *in vitro* (GSH-reactive compounds) [5, 9, 10, 11, 18]. The importance of GSH in erythrocyte based detoxification mechanism, also appears from the increased ability of erythrocytes overloaded with GSH to conjugate increased amounts of the electrophile 1-chloro-2,4 dinitrobenzene for instance [10].

On the other hand, erythrocytes also contain mixed function oxidase (MFO) activity capable of generating GSH-reactive compounds [3]. Erythrocytes are for instance known to metabolize styrene into styrene oxide [3,25,35], benzo(a)pyrene into quinones [19], heterocyclic amines into intermediates with mutagenic activity [7], to hydroxylate aniline [23], and to perform oxidative demethylation on methylaniline with concomittant release of formaldehyde [32]. Oxyhemoglobin is thought to be responsible for this mixed function oxygenase activity [3].

For most compounds the activity of the electrophilic metabolite formation is much higher in the liver [14]. Electrophilic metabolites generated by the liver may be detoxified in turn by the hepatocyte. However, electrophilic metabolites can also be excreted, enter the erythrocytes in the sinusoids, and deplete erythrocyte glutathione (GSH-reactive metabolites).

In the present investigation, attention was paid to the interplay between the bio-activation and inactivation functions of liver and blood. The main questions, approached *in vitro*, were as follows:

- What is the importance of bio-activating and bio-inactivating potency of red blood cells in comparison with the hepatic capacity?
- Are erythrocytes accessible for GSH-reactive electrophilic compounds?
- Is the outer cell membrane of hepatocytes a barrier for reactive metabolites formed intracellularly, or occurs intra-hepatically an effective detoxification?

We developed an incubation system using different combinations of erythrocytes, hemolysate and aqueous GSH solution with rat liver microsomes or intact hepatocytes to imitate the physiological *in vivo* situation as good as possible. An outline of the various experimental conditions studied in the present investigation is depicted in Figure 3.1.

Direct reactivity of the substrate (X) against GSH was tested by means of incubations with aqueous GSH solutions (arrow 1, Figure 3.1). Metabolic activity of erythrocytes for the test substrates (mixed function oxidase (MFO) plus glutathione S-transferase (GST)) was tested through comparison of incubations with aqueous GSH solution and incubations with hemolysate (arrow 2). The influence of erythrocyte integrity on uptake and metabolism of the test compounds was tested by comparison of incubations with hemolysate and erythrocytes (arrow 3). This influence may comprise of influences of the membrane on uptake and, for example, of differences in reductive capacity due to higher oxygen exposure of the hemolysates.

Biotransformation of the test compound by means of microsomal activating system (MAS) into glutathione-reactive metabolites was tested through incubations with aqueous GSH solution with (arrow 1') and without (arrow 1) MAS. These incubations were compared with incubations in which erythrocytes and MAS were combined (arrow 3').

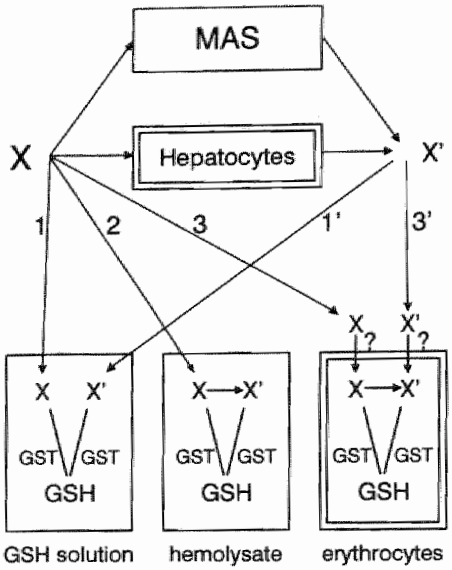

Figure 3.1: An outline of the incubations performed in this study. The picture is explained in the text.

Biotransformation of the test compound by hepatocytes (both phase I and phase II activity) was tested through depletion of hepatocyte GT. The ability of GSH-reactive metabolites to pass the hepatocyte membrane was also tested. Therefore, after incubation of substrate with hepatocytes, the intact cells were removed by centrifugation and the supernatant was incubated again with GSH (arrow 1'). Co-incubations of hepatocytes and erythrocytes (arrow 3') were used to test whether metabolites generated and excreted by hepatocytes are able to enter the erythrocyte and deplete erythrocyte GT.

The compounds we tested can be divided in direct GSH-reactive compounds (iodoacetamide (IAcA), N-ethylmaleimide (NEM), diethyl maleate (DEM)) and compounds which need further metabolisation into GSH-reactive metabolites (cyclophosphamide (CP), 3-hydroxyacetanilide (3-HAA), 2-methylfurane (2-MF) [12]). Two pesticides (monuron (MN) and monulinuron (MLN)) were tested because their metabolite (4-chloroaniline) is known to generate hemoglobin adducts [29]. As the glutathione sulfhydryl group is more sensitive to alkylation than the hemoglobin sulfhydryl group [9], MN and MLN might also affect glutathione concentrations.

In incubations with IAcA, NEM and DEM, reduced glutathione (GSH) was determined. The sum of both reduced and oxidized glutathione (= total glutathione (GT)) was determined when CP, 3-HAA, 2-MF, MN and MLN were tested. The

loss of glutathione (GSH or GT depletion) as a consequence of the incubation with a test compound was used as a measure for the amount of GSH-reactive metabolite.

Materials and Methods

Chemicals

Reduced glutathione (GSH), Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), glutathione reductase (EC 1.6.4.2), β-glucuronidase/arylsulfatase (EC 3.2.1.31/EC 3.1.6.1), Glucose-6-phosphate (G6P), NADP, NADH, cyclophosphamide (CP; CAS: 6055-19-2) and 3-hydroxyacetanilide (3-HAA; CAS: 621-42-1) were obtained from Sigma (St. Louis, USA). Diethyl maleate (DEM), N-ethylmaleimide (NEM) and iodoacetamide (IAcA) were from Janssen Chimica (Beerse, Belgium). 2-Methylfurane (2-MF; CAS: 534-22-5) was from Merck (Darmstadt, Germany), and monuron (MN; CAS: 150-68-5) and monolinuron (MLN; CAS: 1746-81-2) were from Riedel-de Haën (Seelse, Germany). Collagenase B (from Clostridium histolyticum) was obtained from Boehringer (Mannheim, Germany). Water was demineralized and microfiltrated before use. All chemicals were of analytical grade quality.

Animals

Male Brown Norway rats (BN/M) of about 250 grams were used. Housing was as previously described [26]. When liver microsomes from rats pretreated with phenobarbital (PB microsomes) were used, phenobarbital^{TM} was added to the drinking water (1 g·l^{-1}) during one week prior to the preparation of microsomes.

Preparation of microsomal activating system and the isolation of hepatocytes

The preparation of microsomal activating system was described previously [26]. The procedure for the isolation of hepatocytes was based on the methods described by Berry and Friend [4] and by Seglen [30]. A calcium and magnesium free buffer solution (100 mM NaCl, 7 mM KCl, 39 mM HEPES and 15 mM NaH_2PO_4 (pH adjusted to 7.4 with NaOH)) was perfused (30 ml·min^{-1}) in a single pass way through the isolated rat liver during 20 minutes. Next, a collagenase containing buffer (0.05 gram·100 ml^{-1}), composed of 67 mM NaCl, 7 mM KCl, 100 mM HEPES and 5 mM $CaCl_2 \cdot 2H_2O$ (pH adjusted to 7.4 with NaOH), was perfused in a recirculating way during 5 minutes. Hepatocytes were isolated from the liver, filtrated (100 μM) and washed three times in a Krebs-Henseleit buffer (pH 7.4; 4 minutes, centrifuge force: 25×g). The viability of the cells (more than 90 %) was determined with the Trypan-blue exclusion test [16].

Preparation of erythrocytes and hemolysate

Human blood was obtained from the Red Cross Blood Bank "Zuid Limburg", collected in sterile vacuum EDTA vessels, checked for viral infections, and kept overnight at 4°C. Erythrocytes of at least 4 persons were pooled, washed three times using saline and diluted in 100 mM Na_2HPO_4/KH_2PO_4 buffer (hemoglobin (Hb) concentration 100 $g \cdot l^{-1}$, pH 7.4) in incubations with microsomes, or in Krebs-Henseleit buffer (Hb concentration 27 $g \cdot l^{-1}$, pH 7.4) in incubations with hepatocytes.

Hemolysate was prepared from packed erythrocytes by dilution with ice-cold water (1:2). The cellular debris was removed by centrifugation (5 minutes, 2000×g).

Incubations

Each experiment consisted of incubations performed in triplicate. For direct GSH-reactive compounds a single experiment was done. Experiments with CP, 3-HAA, 2-MF, MN and MLN and aqueous GSH solution, hemolysate and erythrocytes with or without microsomes were repeated three times on different days (total: 3×3). Co-incubation experiments of erythrocytes and hepatocytes were also repeated three times. Incubations with hepatocytes without erythrocytes, were based on a single experiment for CP, and repeated three times for 3-HAA.

Incubations of direct GSH-reactive compounds with aqueous GSH or whole blood

Whole blood (1.5 ml) or an equal volume of aqueous GSH solution in phosphate buffered saline (pH=7.3), were incubated with the test compounds (50 μl). IAcA and NEM were dissolved in phosphate buffered saline, and DEM in ethanol. Aqueous GSH solution (initial concentration 500 μM) was incubated with IAcA, NEM and DEM for 1 hour in a shaking waterbath (210 revolutions per minute) at 37°C. Human blood (initial GSH concentrations 860, 720 and 820 μM) was also incubated with IAcA, NEM and DEM respectively (37°C, 210 revolutions per minute). After an incubation period of 15 minutes, the GSH concentration was measured. Final concentrations of 0, 0.5, 1.0 and 2.0 mM IAcA, NEM and DEM were tested.

Incubations with hemolysate

To verify whether substances without direct activity to GSH (CP, 3-HAA, 2-MF, MN and MLN) were metabolically activated by erythrocytes themselves, GT depletion in hemolysate was tested (initial GT concentration 235 (standard error of the mean (SE)=20 μM). After pre-incubating hemolysate for 5 minutes at 37°C in a shaking waterbath (80 revolutions per minute), 1 ml test compound

(final concentrations 0, 0.5, 1.0 and 2.0 mM) was added. After 1 hour incubation the reaction was stopped by the addition of an equal volume of 5 % (w/v) trichloroacetic acid (TCA).

Co-incubations of aqueous GSH solution or erythrocytes with and without microsomal activating system

Erythrocytes (final Hb concentration 23 $g \cdot l^{-1}$, final GT concentration 110 (SE=4) μM) or aqueous GSH solutions (final concentrations of 440 (SE=5) μM were suspended in 100 mM Na_2HPO_4/KH_2PO_4 buffer (final volume 3 ml). For 2-MF the initial GT concentration was 300 (SE=5) μM. Incubations were performed both with and without microsomal activating system. Both normal and PB microsomes were tested. The complete test system without test substance was pre-incubated for 5 minutes at 37°C in a shaking waterbath (80 revolutions per minute). Thereafter, 1 ml test substance (final concentration between 0 and 2 mM) was added and incubated for 1 hour. For CP and 3-HAA, separate experiments were performed for test compound concentrations between 0 and 0.5 mM and between 0.5 and 2.0 mM. After 1 hour incubation the samples were treated as previously described [26]. Finally, GT concentrations were measured.

Incubations with hepatocytes

Hepatocytes ($4.4 \cdot 10^6$ cells$\cdot ml^{-1}$) plus 3-HAA or CP solutions (final concentrations between 0 and 2.0 mM) were incubated in Krebs-Henseleit buffer (pH 7.4) during 1 hour (incubation volume 1.5 ml, 37°C) in a shaking water bath (80 revolutions per minute). After the incubation period, 200 μl TCA (8 %) was added to an equal volume of hepatocyte suspension and GT was determined. 100 μl Hepatocytes were lysed with ice cold water (1:6.7 v/v) and frozen until protein determination (-70°C). The rest of the sample was centrifugated (5 min, 25×g) and 1 ml supernatant plus 200 μl GSH solution (final concentration between 100 and 400 μM) was incubated again under the same conditions. In order to liberate possible conjugated metabolites, β-glucuronidase/arylsulfatase was added (final activity of 650 $U \cdot ml^{-1}$). The pH was adjusted to 6.2 by addition of 175 μl 3 M KH_2PO_4.

Co-incubations of erythrocytes and hepatocytes

Co-incubations of erythrocytes and hepatocytes were performed in transwells^{TM} (Costar, Cambridge, England), that are composed of a well in which a smaller well (transwell) can be placed containing a porous membrane (0.4 μm). Erythrocytes (2.6 ml, final Hb concentration 27 $g \cdot l^{-1}$) were pipetted into the well, and the hepatocytes (1 ml, $6.6 \cdot 10^6$ cells$\cdot ml^{-1}$) plus 3-HAA (0.5 ml, final concentration between 0 and 5 mM) were added to the transwell. After an incubation period of

1 hour, the erythrocytes were pipetted into plastic vials and packed by centrifugation. The rest of the procedure was the same as in co-incubations of microsomes and erythrocytes. Finally, GT concentrations were determined.

Analytical procedures

GSH was determined, essentially as described by Anderson [2]. Sample preparation was described previously [9]. GT in the TCA supernatants was determined after dilution (9 fold) with buffer by the cyclic oxidation reduction method described by Anderson [2]. Lactate dehydrogenase (LDH) activity was determined using NADH consumption during pyruvate transformation to lactate [24]. Microsomal and hepatocyte protein content was determined according to Lowry *et al.* [20]. Hemoglobin concentrations were determined by measuring cyanmethemoglobin according to Van Kampen and Zijlstra [17].

Statistical analysis

GT depletion caused by CP, 3-HAA, 2-MF, MN and MLN was analyzed separately for both incubations with normal and PB microsomes by linear regression, using a model including day of experiment and concentration as explanatory variables. Using that model we tested whether slopes of GT depletion versus concentration were significant for each of the metabolizing systems, correcting for the day of the experiment.

Results

Incubations without addition of metabolic activating system

Depletion of GSH by IAcA, NEM and DEM or depletion of GT by CP, 3-HAA and 2-MF in incubations without metabolic activating system (MAS), is shown in Figures 3.2, 3.3 and 3.4 for aqueous GSH solutions, erythrocytes and hemolysate respectively.

In incubations with *aqueous GSH solution* (initial GSH concentration 500 μM), GSH was depleted by the direct GSH-reactive compounds IAcA, NEM and DEM. NEM was the most reactive compound as GSH was almost fully depleted when 0.5 mM NEM was tested, followed by IAcA which was almost fully depleted at a concentration of 1.0 mM. DEM was the least reactive compound.

The incubations with *erythrocytes* and IAcA, NEM and DEM showed that erythrocyte GSH could also be depleted by these compounds, indicating that these GSH-reactive electrophiles could pass the erythrocyte membrane. IAcA was the

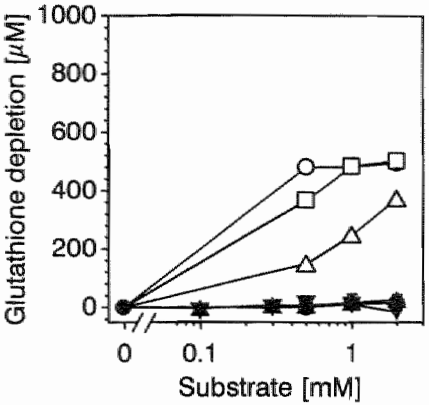

Figure 3.2: Incubations with aqueous GSH solution. GSH depletion in incubations with IAcA □, NEM ○ and DEM △ and GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. The mean of the GSH or GT depletion plus the SE of the GT depletion is shown (interexperimental variation). For some of these incubations the SE falls within the datapoints.

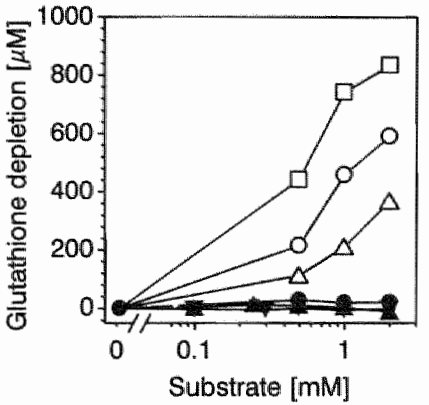

Figure 3.3: Incubations with erythrocytes. GSH depletion in incubations with IAcA □, NEM ○ and DEM △ and GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. The mean of the GSH or GT depletion plus the SE of the GT depletion is shown (interexperimental variation). For some of these incubations the SE falls within the datapoints.

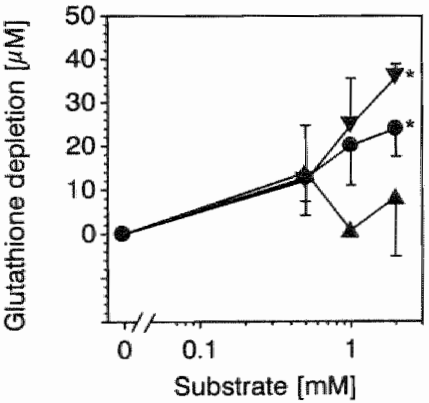

Figure 3.4: Incubations with hemolysate. GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. The mean and the SE of the GT depletion is shown (interexperimental variation). The GT depletion was concentration dependent both for 3-HAA and 2-MF ($^* p \leq 0.01$ in both cases).

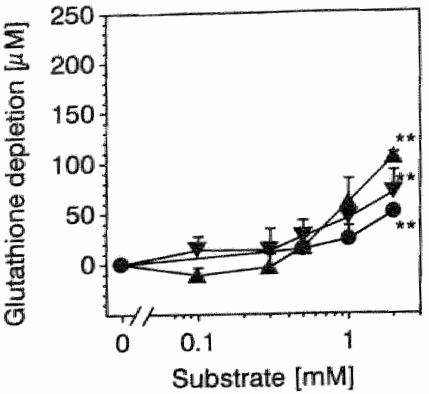

Figure 3.5: Incubations with aqueous GSH solution and microsomes of untreated rats. GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. The mean and the SE of the GT depletion is shown (interexperimental variation). The GT depletion was concentration dependent for all compounds tested (** $p \leq 0.001$ in all cases).

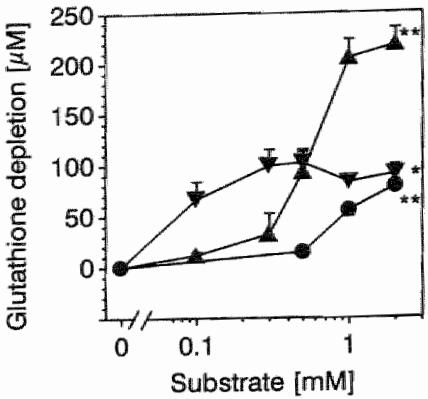

Figure 3.6: Incubations with aqueous GSH solution and microsomes of PB pretreated rats. The mean and the SE (interexperimental variation) of the GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. In some cases the SE falls within the datapoints. The GT depletion was concentration dependent for all compounds (* $p \leq 0.01$ for 3-HAA; ** $p \leq 0.001$ for CP and 2-MF).

most reactive compound in these incubations as 97 % of the initial GSH concentration was depleted at a IAcA concentration of 2 mM, followed by NEM and DEM (82 % and 45 % resp.; 2 mM test compound).

GT was not depleted in incubations with *aqueous GSH solution* or *erythrocytes* when CP, 3-HAA, 2-MF, MN and MLN were tested.

In incubations with *hemolysate*, 3-HAA and 2-MF caused a concentration dependent GT depletion ($p \leq 0.01$ in both cases). The amount of GT depleted was 18 μmoles·mmol^{-1} 3-HAA and 12 μmoles·mmol^{-1} 2-MF.

Incubations with microsomal activating system

In incubations with *aqueous GSH solution* and *normal microsomes* (Figure 3.5), GT was depleted in a concentration dependent way by CP, 3-HAA and 2-MF ($p \leq 0.001$ in all cases). The amount of GT depleted by CP was 48 μmoles·mmol^{-1} CP, by 3-HAA was 36 μmoles·mmol^{-1} 3-HAA and by 2-MF was 25 μmoles·mmol^{-1} 2-MF.

When CP, 3-HAA and 2-MF were tested in incubations with *aqueous GSH solution* and *PB microsomes* (Figure 3.6), higher GT depletions were found compared

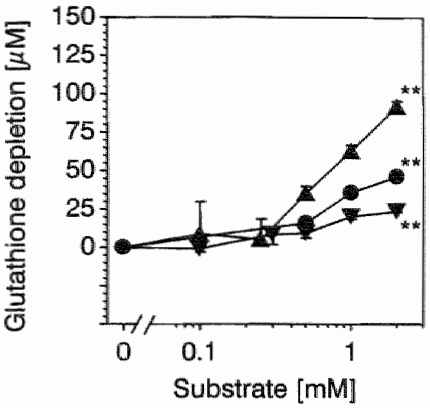

Figure 3.7: Incubations with erythrocytes and microsomes of untreated rats. GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. The mean and SE of the GT depletion is shown (interexperimental variation). For some of these incubations the SE falls within the datapoints. The GT depletion was concentration dependent for all compounds tested ($^{**}p \leq 0.001$).

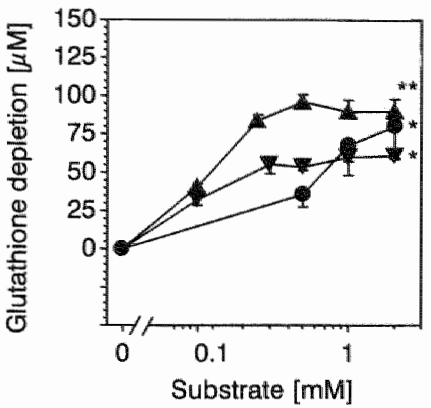

Figure 3.8: Incubations with erythrocytes and microsomes of PB pretreated rats. GT depletions in incubations with CP ▲, 3-HAA ▼ and 2-MF ● are shown. The mean and SE of the GT depletion is shown (interexperimental variation). The GT depletion was concentration dependent for all compounds tested ($^{*}p \leq 0.01$ for 3-HAA and 2-MF; $^{**}p \leq 0.001$ for CP).

to incubations with normal microsomes. GT depletions were concentration dependent for all three compounds tested ($p \leq 0.01$ for 3-HAA; $p \leq 0.001$ for CP and 2-MF). The amount of GT depleted by CP and was 92 μmoles·mmol^{-1} CP, and by 2-MF 41 μmoles·mmol^{-1} 2-MF. For 3-HAA, the amount of GSH-reactive metabolite formed was maximal at a 3-HAA concentration of 0.3 mM. At this 3-HAA concentration the initial GSH concentration was about 25 % depleted.

GT was not depleted when MN or MLN were tested in incubations with aqueous GSH solution, irrespective of the kind of microsomes used.

In incubations with *erythrocytes* and *normal microsomes* (Figure 3.7), GT was depleted in a concentration dependent way by CP, 3-HAA and 2-MF ($p \leq 0.001$ in all cases). The amount of GT depleted in incubations with CP was 46 μmoles·mmol^{-1} CP, with 3-HAA was 11 μmoles·mmol^{-1} 3-HAA, and with 2-MF was 23 μmoles·mmol^{-1} 2-MF.

In incubations with *erythrocytes* and *PB microsomes* (Figure 3.8), GT was also depleted in a concentration dependent way when CP, 3-HAA and 2-MF were tested ($p \leq 0.01$ for 3-HAA and 2-MF; $p \leq 0.001$ for CP). Considering CP, all glutathione was consumed at 0.5 mM CP. The amount of GT depleted in incubations with CP was 188 μmoles·mmol^{-1} CP regarding CP concentrations

up to 0.5 mM. 3-HAA caused a GT depletion of 30 μmoles·mmol^{-1} 3-HAA and 2-MF a GT depletion of 39 μmoles·mmol^{-1} 2-MF.

Again, GT was not depleted when MN or MLN were tested in incubations with human erythrocytes, irrespective of the kind of microsomes used.

GT was only depleted in incubations with aqueous GSH solution or erythrocytes when both microsomes (normal or PB) and NADPH regenerating system were added.

Incubations with hepatocytes

viability of hepatocytes

The viability of hepatocytes was tested by measurement of LDH leakage into the medium [16]. LDH leakage was low during the first 50 minutes of the incubation period and increased thereafter (data not shown). The viability of the hepatocytes was not affected by 3-HAA or CP (final concentration 2 mM) as the LDH activity in the supernatant was not higher than in controls after a one-hour incubation period (Table 3.1). Incubation of hepatocytes with DEM (final concentration 1 mM, 15 minutes, 37°C) depleted the GT concentration up to 72 % but had no effect on LDH leakage (data not shown).

Table 3.1: Viability of hepatocytes, hepatocyte GT depletion after incubation with 3-HAA and CP and excretion of GSH-reactive metabolites by hepatocytes.

test compound	concentration (mM)	LDH (U·l^{-1})	GT-hep (μmoles·g^{-1} protein)	GT-sup (μM)
3-HAA	0	1616	39 (SE=5)	369
	1.0	1564	n.d.	319
	2.0	1545	32 (SE=3)	294
CP	0	1616	38	141
	1.0	1514	n.d.	n.d.
	2.0	1494	30	135

LDH is the LDH activity (U·l^{-1}) in the incubation medium after a 1-hr incubation period with 3-HAA and CP; GT-hep is the amount of GT in hepatocytes per gram protein (4 g·l^{-1}), after a 1-hr incubation period with 3-HAA and CP; GT-sup is the GT concentration in supernatants of incubations with hepatocytes and 3-HAA and CP, which were incubated again with aqueous GSH solution for 1 hour.

Incubations with hepatocytes without erythrocytes

GT depletion in hepatocytes (GT-hep; Table 3.1) was 7 μmoles·g^{-1} protein when 2 mM 3-HAA was tested and 8 μmoles·g^{-1} protein when 2 mM CP was tested. The decrease in hepatocyte GT concentration caused by 3-HAA was concentration dependent ($p = 0.01$).

The ability of GSH-reactive metabolites to pass the hepatocyte membrane was tested. For this purpose, after incubation of substrate with hepatocytes, the intact cells were removed by centrifugation and the supernatant was incubated again with GSH during one hour under the same conditions. Table 3.1 shows the results of a typical experiment both for 3-HAA and CP. It shows that the GT concentration in the supernatants (GT-sup) decreased as a result of the former incubation of hepatocytes with the test compound, indicating that GSH-reactive 3-HAA and CP metabolites are excreted by the hepatocytes.

Electrophiles generated by hepatocytes may also be detoxified by conjugation in the hepatocyte and excreted into the medium. The presence of glucuronide conjugates was tested by means of the addition of β-glucuronidase/arylsulfatase to the medium of the second (GSH containing) incubation, so that GSH may be able to react with the released metabolite. However, addition of β-glucuronidase/arylsulfatase did not lead to more GT depletion.

Co-incubations of erythrocytes and hepatocytes

Erythrocyte GT concentration was not affected when erythrocytes and hepatocytes were co-incubated. Addition of 3-HAA to these incubations (final concentrations up to 5 mM) did not affect erythrocyte GT, neither when normal hepatocytes were used nor when hepatocytes from phenobarbital pretreated rats were tested.

Pre-incubation of hepatocytes with DEM, which lead to a 72 % GT depletion in the hepatocytes, did also not affect the erythrocyte GT concentration. As electrophiles may be sulfated in the hepatocyte, hepatocytes were isolated and incubated with sulfate-free buffer. Incubation in sulfate-free medium is known to affect sulfation capacity [8]. However, no effect on erythrocyte GT was found.

Discussion

Most cell types are more or less able to (de)toxify foreign chemicals. The hepatocyte is known as a metabolically very active cell and erythrocytes have some metabolic activity also [3]. Foreign compounds, toxified by hepatocytes by means of cytochrome P-450 activity, may be detoxified by phase II metabolism (e.g. glucuronidation, sulfation, glutathione conjugation). This may lead to

a shortage of hepatocyte phase II conjugation factors like UDP-glucuronic acid, 3'-phosphoadenosine-5'-phosphosulfate and reduced glutathione (GSH). Conjugated and/or unconjugated metabolites may be released by the hepatocyte into the sinusoids. This means that erythrocytes can be exposed to these metabolites. When the metabolites are able to pass the erythrocyte membrane, the biochemical functions of the erythrocyte may be affected.

In the present investigation, we developed a set of *in vitro* test-systems (Figure 3.1), that may gain an insight into the interplay between the bio-activation and inactivation functions of liver and blood, using glutathione depletion as dependent variable. A comparable method was described by Tingle and Park [34], who found methemoglobin formation in human erythrocytes when dapsone was tested in incubations with rat liver microsomes and human erythrocytes. To approach the physiological *in vivo* situation more closely, we tested whether hepatocytes are able to generate GSH-reactive metabolites from CP or 3-HAA, and the ability of 3-HAA metabolites to deplete erythrocyte GT. As a large variety of variables (beside glutathione depletion) can be tested, the test-systems may extend the current set of techniques. GSH is an efficient scavenger of reactive electrophilic substances, especially in combination with glutathione dependent enzymes [6, 21, 22, 31]. Because of this property, a decrease of the GSH concentration (glutathione depletion) caused by the test compound, has been used as a measure for the amount of GSH-reactive compounds in the incubate [11, 12, 13].

IAcA, NEM and DEM are direct GSH-reactive compounds which need no metabolic activation [9]. In incubations with aqueous GSH solution and in incubations with erythrocytes, GSH was depleted. This means that these test compounds are able to pass the erythrocyte membrane and deplete erythrocyte GSH. As GSH was also depleted in incubations with the GST lacking aqueous GSH solution, catalysation of the conjugation reaction by erythrocyte GST is not needed for these highly reactive compounds. CP, 3-HAA, 2-MF, MN and MLN did not influence GT concentrations (GT is the sum of both reduced and oxidized glutathione) of aqueous GSH solutions or erythrocytes when no external metabolizing system was added, indicating that these compounds have no direct action on GSH.

Both 3-HAA and 2-MF depleted GT in hemolysate but not in incubations with aqueous GSH solution or erythrocytes. The results indicate that a factor in the hemolysate is capable to metabolize 3-HAA and 2-MF resulting in GSH conjugation. This metabolic activity was not explained by mere GST catalyzed glutathione conjugation to the parent compounds as addition of glutathione S-transferase π (3.2 U·ml^{-1}) to the incubations with aqueous GSH solution and 3-HAA or 2-MF, had no effect on the GT concentration (data not shown).

Only when rat MAS was added to incubations of CP, 3-HAA or 2-MF and aqueous GSH solution or erythrocytes, GT was depleted. GT depletions were concentration dependent when normal or PB microsomes were used. Addition of NADPH

was a requirement. This indicates that metabolites of CP, 3-HAA and 2-MF are generated by MAS and that these metabolites are able to enter the erythrocyte. For all three compounds tested, GT depletions were higher in incubations with aqueous GSH solution than in erythrocytes when normal microsomes were tested. This may indicate that the erythrocyte membrane acts as a barrier for metabolites of CP, 3-HAA and 2-MF, generated by microsomes. If the erythrocyte membrane would limit diffusion to less than 80 nmoles·h^{-1}·m^{-2} membrane, GT depletion would be less than 10 % during the assay period and therefore probably not be detectable. (In 200 μl erythrocytes the amount of GSH is 300 nanomoles. The amount of GSH-reactive metabolite needed to deplete 10 % of this GSH pool would be 30 nanomoles. The membrane surface of 200 μl erythrocytes is 0.36 m^2 [15]). Both in aqueous GSH solutions and in erythrocytes, GT depletion by CP, 3-HAA and 2-MF was higher with PB pretreated microsomes than with normal microsomes. This means that the metabolite generation of CP, 3-HAA and 2-MF is mediated by the phenobarbital inducable MAS. When MN or MLN were tested in incubations with aqueous GSH solution or erythrocytes, the quantity of reactive metabolites formed in the present study was insufficient to give a detectable GT depletion. Addition of normal or PB microsomes had no influence. MN and MLN are known to give (4-chloroanilin) Hb adducts in rats [29]. Presence of reactive MN or MLN metabolites in erythrocytes would not only lead to hemoglobin adducts but also to glutathione conjugation, as GSH is more reactive than the nucleophilic sites in Hb [9].

Hepatocyte GT decreased 7–8 μmoles·g^{-1} protein in incubations with 2 mM CP or 3-HAA. The protein concentration of hepatocytes is about 200 g·l^{-1}, which means that the GT depletion *in* hepatocytes is about 1.5 mM at 2 mM CP or 3-HAA. To verify whether a substantial fraction of the metabolites formed was excreted by the hepatocytes, the supernatants of the 3-HAA or CP hepatocyte incubations were reincubated with GSH during one hour under the same conditons. The GT depletion in the supernatant of incubations with 3-HAA (2 mM) was 75 μM. When the volumes of hepatocytes and supernatants (22 μl and 1.5 ml respectively) are taken into account, the total amount of GSH-reactive 3-HAA metabolite *within* the cells and *excreted* into the supernatant can be calculated to be 33 and 113 nanomoles respectively, when we assume that the metabolites are equally distributed over hepatocytes and supernatant. These results indicate that a substantial fraction (77 %) of the GSH-reactive 3-HAA metabolites formed by the hepatocytes was excreted. For CP (2 mM), only 21 % of the GSH-reactive metabolites was excreted by the hepatocytes (GT depletion in the supernatant=6 μM). For this reason 3-HAA was used in co-incubations of hepatocytes and erythrocytes. For technical reasons enforced by the geometry of the "transwells", the excreted metabolite concentration was diluted even further in the co-incubations of hepatocytes and erythrocytes (incubation volume 4.1 ml instead of 1.5 ml), leading to a calculated 3-HAA metabolite concentration of

28 μM outside the erythrocytes at the end of the incubation. If we suppose that all of this substance enters the erythrocyte during the experiment, a detectable depletion of about 30 % would have occurred. However, in co-incubations of hepatocytes and erythrocytes no effect of 3-HAA on erythrocyte GT was found. In practice uptake limitation may therefore be an important factor. Assuming that metabolite excretion of the hepatocytes is linear with time, the mean metabolite concentration outside the erythrocytes was 14 μM. Using Fick's law we can calculate that the needed mass transport (80 nmoles$\cdot$h$^{-1}\cdot$m^{-2}, see above) can only be reached with a permeability coefficient of at least $0.7\cdot10^{-8}$ cm$\cdot$s^{-1}. For comparison, known values are about 10^{-7} cm$\cdot$s^{-1} for glucose and 10^{-10} cm$\cdot$s^{-1} and lower for ions [1]. This indicates that an effective depletion would only be detectable within one hour when the diffusion uptake speed of the metabolites was at least about 10 % of the uptake speed of glucose.

To increase the excretion of GSH-reactive metabolites by the hepatocytes, hepatocytes were pre-incubated with diethyl maleate in order to deplete their glutathione store. While this treatment lead to over 70 % depletion of GT in the hepatocytes, no effect of this treatment on erythrocyte GT was found. Rashed *et al.* described that 3-HAA and 3-HAA metabolites generated by oxidative metabolism, are sulfated and glucuronidated in mice [28]. In their study urinary glucuronides and sulfates of 3-HAA and its metabolites accounted for 80 % of the administered dose. Since sulfation by isolated hepatocytes can extensively be reduced when no inorganic sulfate is administered [8], this treatment may increase the amount of unconjugated reactive metabolites in the hepatocytes and, in case of excretion, in the supernatant. However, isolation of hepatocytes with sulfate-free buffer solutions, and incubation of hepatocytes and erythrocytes in sulfate-free Krebs buffer, did not deplete erythrocyte GT when 3-HAA was tested. Addition of β-glucuronidase/arylsulfatase to incubations composed of GSH and supernatant which was previously incubated with hepatocytes and 3-HAA, had also no influence on GT depletions.

In summary, the direct GSH-reactive compounds IAcA, NEM and DEM were able to enter the erythrocyte and deplete the erythrocyte GSH store. CP, 3-HAA and 2-MF only depleted erythrocyte GT when MAS was added indicating that GSH-reactive metabolites were formed which were able to enter the erythrocyte. Hepatocytes also metabolised 3-HAA and CP into GSH-reactive metabolites. They were able to excrete a fraction of these GSH-reactive metabolites into the supernatant. For 3-HAA, no erythrocyte GT depletion was found in co-incubations of hepatocytes and erythrocytes which may be explained by uptake limitation of the GSH-reactive metabolites into the erythrocytes.

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Chapter 4

Toxicokinetics of dimethylacetamide (DMAc) in rat isolated perfused liver

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Summary

Dimethylacetamide (DMAc) is a skin-penetrating solvent able to induce hepatic damage after chronic exposure. Previous research has indicated that metabolism may be saturated at its present TLV/TWA (10 ppm). Biological monitoring of monomethylacetamide (MMAc), the primary metabolite of DMAc, might therefore underestimate exposure to DMAc and related health hazards. We used the recirculating perfusion technique in isolated rat liver to evaluate DMAc metabolism. Medium concentrations starting at about 30, 50, 100 and 275 μM, respectively, were tested. Perfusate samples were taken regularly and analyzed for DMAc; pharmacokinetic parameters (extraction ratio and clearance) were calculated for each perfusion. Inlet DMAc concentrations were calculated and concentration groups divided in 16, 36, 70, 160, 225 μM. The extraction ratio of the 16 μM group differed significantly from the other concentration groups tested. DMAc metabolism was saturated at a DMAc concentration of 36 μM. Extraction ratios were unaffected when cimetidine, an inhibitor of cytochrome P-450 activity, was added to the perfusion medium or when cimetidine-pretreated animals were used. DMAc clearance was 2.20 ml·min^{-1} at a medium concentration of about 36 μM. Extrapolation of the observed (rat)liver clearance to man showed that airborne concentrations of 18 ppm would, under the presumptions used, lead to saturated metabolism of DMAc; however, saturation at even lower concentrations could not be excluded.

Introduction

Dimethylacetamide (DMAc) is a colourless solvent which is used in the production of synthetic fibres, plastics, resins and gums and as an adjuvant for veterinary purposes. Its acute toxicity is low, with an oral LD_{50} of 4 g·kg^{-1} in the rat [6]. The primary effects occurring at high dosage are respiratory failure and atonic state of the muscles. The most important effect considered for standard setting is the occurrence of hepatotoxicity at relativily low chronic exposures [7, 10]. DMAc's liver toxicity might be related to specific biotransformation into (intermediate) reactive metabolites such as N-hydroxymethyl N-methylacetamide which was detected in urine of rats treated subcutaneously with ^{14}C labeled DMAc [16]. In humans the only metabolite observed *in vivo* was monomethylacetamide (MMAc) [5, 11, 13]. However, data with regard to the extent of its metabolism are conflicting, and impede rational standard setting or control by biological monitoring.

As exposure at the TLV and at higher levels produce almost the same concentrations of urinary MMAc (Figure 4.1), it was previously suggested [5, 9, 8] that DMAc metabolism might be saturated at its current TLV/TWA (10 ppm).

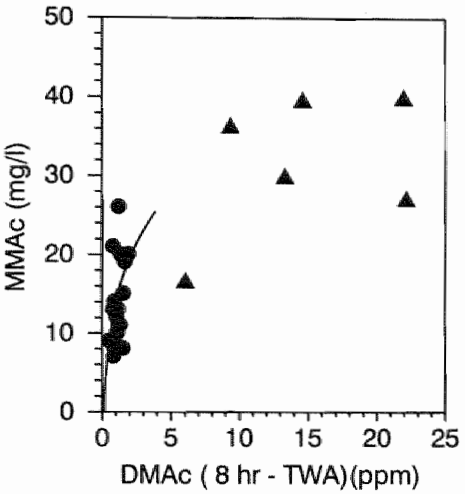

Figure 4.1: Plot of urinary MMAc excretion ($mg \cdot l^{-1}$) *versus* DMAc exposure (ppm) in workers during 8-hr workshifts. Data were taken from Borm *et al.* (▲) [5] and Kennedy *et al.* (●) [11]. MMAc concentrations were determined in post-work urinary samples. As urinary volumes were not determined in both studies, we assumed that urine production did not vary to a great extent. The data indicate saturation of MMAc excretion/DMAc metabolism around its current threshold limit value (10 ppm).

Since the liver is both the primary site of DMAc metabolism and the ultimate target for its *in vivo* toxicity, we set out to study DMAc metabolism and kinetics in isolated perfused rat liver. The ultimate purpose was to evaluate our data with respect to possible saturation of metabolism.

Dimethylformamide (DMF), a homologue of DMAc, is N-demethylated *in vitro* and *in vivo* by microsomal enzymes [2]. To evaluate a similar conversion for DMAc we tested whether pretreatment of the rats with cimetidine, an inhibitor of cytochrome P-450 dependent N-dealkylation [4, 17], or addition of cimetidine to the perfusion medium inhibits DMAc clearance.

Methods

Chemicals

Dimethylacetamide (DMAc), monomethylacetamide (MMAc) dimethylformamide and 4-dimethylaminoantipyrine were obtained from Janssen Chimica (Beerse, Belgium), bovine serum albumin (BSA) from Organon Teknika (Boxtel,

The Netherlands), lactate dehydrogenase (LDH, from rabbit muscle) EC 1.1.1.27 and glucose 6-phosphate dehydrogenase EC 1.1.1.49 from Boehringer (Mannheim, FRG) and cimetidine, NADP and glucose 6-phosphate from Sigma (St. Louis, U.S.A.). Water was demineralized and filtrated before use. All other chemicals were of analytical grade quality.

Animals

Male Wistar rats (Bor: WISW (SPFCpb)) weighing 250–280 g were used; about 12 hr before the start of an experiment animals were deprived of food (SRMA 1210, Hope Farms), but had access to water. Cimetidine pretreated rats (75 $mg \cdot kg^{-1}$ per day per rat) were injected i.p. with cimetidine in saline during three days prior to the experiment. Phenobarbital was given orally (1 $g \cdot l^{-1}$) during one week prior to the preparation of phenobarbital-induced microsomes.

Preparation of microsomes

Animals were anaesthetized using pentobarbital (Narcovet ^{TM}; 0.2 $ml \cdot 100\ g^{-1}$ rat). Livers were removed and homogenized in three volumes of ice-cold phosphate buffer (50 mM and pH 7.4), using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged at 9000×g for 20 minutes at 4°C. The microsomal fraction was sedimented from the supernatant by 40 minutes centrifugation at 110000×g and 4°C. The pellet was resuspended in the same ice-cold buffer and diluted so that the microsomal fraction of 3 g liver was diluted in 1 ml buffer.

Microsomal incubations

The inhibitory effect of cimetidine on cytochrome P-450 activity [4, 17], was evaluated by determination of microsomal dimethylaminoantipyrine (DMAP) demethylation activity. The incubations were performed according to Bast [3]. However, the microsomal protein content was 1 $mg \cdot ml^{-1}$, the semicarbazide concentration was 1 mM and the glucose 6-phosphate dehydrogenase activity was 1 $IU \cdot ml^{-1}$. Microsomes of animals pretreated with cimetidine and microsomes to which cimetidine was added during the incubation (1 mM) were both tested. When no cimetidine was added to incubations with phenobarbital pretreated microsomes (n=2), formaldehyde concentrations were 127.0 μM compared to 25.5 μM when cimetidine was added to the incubations (n=2). Pretreatment of animals with cimetidine had no effect on microsomal DMAP metabolism.

Perfused rat liver: preparation and experiments

The liver perfusion and all surgical procedures were done according to Meijer *et al.* [14]. Bicarbonate buffered Krebs-solution with 1 % bovine serum albumin (BSA) and 11 mM glucose was filtered (0.2 μm) and used as perfusate.

DMAc was added to the perfusion medium before the start of the perfusion. DMAc concentrations of about 30, 50, 100 and 275 μM were tested. Medium flow was kept constant during perfusion. Flow was regulated by changing hydrostatic pressure. Bile and perfusate samples were taken every 5 minutes after an initial stabilization period of 10 minutes. Bile secreted during perfusion was expressed as milligram bile per minute per gram liver. Liver damage was assessed through LDH-activity (EC 1.1.1.27) in perfusate samples, using the NADH consumption during pyruvate transformation to lactate [12]. Albumin was added to the perfusate samples (final concentration 3 %) which were then stored at -80°C until determination. Histological preparations (haematoxilin-eosin staining) of a liver sample were made 1 hour after the initiation of all experiments and examined by light microscopy. The liver was homogenized using a Potter-Elvehjem homogeniser and assayed for DMAc.

Analytical procedures

Samples of liver perfusate were saturated with NaCl and extracted with an equal volume of acetonitrile. After addition of an internal standard (DMF; final concentration 36 μM), 0.2 μl of the extracted sample was analyzed for DMAc and MMAc by gas chromatography. A Carlo Erba 5300 MS gas chromatograph fitted with a DB-Wax 20 M column (internal diameter 0.326 mm; length, 30 m; film thickness, 0.5 μm) and an on-column injection unit was used. Column temperature was kept at 120°C, and the detector (flame ionization) temperature was set at 240°C. Helium was used as a carrier-gas at a flow rate of 3 ml·min^{-1}. Under these conditions the retention times for DMAc and MMAc were 5.2 and 12.2 minutes, respectively. Peak-height ratios of DMAc and dimethylformamide were determined and DMAc concentrations in the perfusate samples were calculated. MMAc concentrations were determined according to the same procedure. Under these conditions the detection limits were 10 and 35 μM for DMAc and MMAc, respectively. To evaluate the experimental error, the coefficient of variation (SD$\times$100 %/mean) for DMAc and MMAc measurements were determined. At the detection limit for DMAc (10 μM) this was 12 %; for concentrations $\geq$ 20 μM 4 %. At the detection limit for MMAc (35 μM) this was 58 %; for concentrations $>$ 35 μM 6 %. Final concentrations in the perfusates were corrected for the decrease in medium volume as a result of sampling (see below).

Calculation of extraction ratios

As samples were taken by redirection of the medium flow from the liver towards the main vessel to a sample vial, the DMAc concentration in the perfusion system was constant during the time that samples were taken, resulting in an undervaluation of the decline in DMAc medium concentration as a consequence of DMAc metabolism by the liver. This would result in an underestimation of the

extraction ratio for DMAc. The incoming DMAc medium concentration (C_{in}) was calculated using the samples of the outcoming DMAc medium concentration (C_{out}), supposing ideal mixture of the outcoming perfusate with the rest of the medium. C_{out} values between the measurements were interpolated assuming exponential decrease. C_{in} values were calculated as:

$$C_{in} = \frac{A_{in}}{V} dA_{in} = (Q_{in}C_{in} - Q_{out}C_{out})dt \tag{4.1}$$

As the DMAc concentration does not change during the time samples were taken (Q_{out}=0), those periods were not used for further evaluation. The volume of distribution was calculated as the DMAc dose divided by the DMAc medium concentration at the start of the perfusion, obtained by linear extrapolation of a log linear concentration *versus* time plot. Extraction ratios (E) were calculated by dividing the difference between C_{in} and C_{out}, and C_{in}:

$$E = \frac{C_{in} - C_{out}}{C_{in}} \tag{4.2}$$

Extraction ratios were integrated during a 40 minutes perfusion period starting 10 minutes after the onset of the perfusion and divided by the time period the integral was taken, to level variance in the calculated extraction ratios.

Calculation of airborne concentrations leading to saturation

DMAc clearance (Cl) was calculated as medium flow times the extraction ratio:

$$Cl = Q \cdot E \tag{4.3}$$

DMAc clearance times DMAc medium concentration (c_1) equals metabolic rate (v):

$$v = Cl \cdot c_1 \tag{4.4}$$

At steady-state the uptake of airborne DMAc equals its metabolic elimination rate and can be calculated by multiplying alveolar ventilation ($\dot{V}_{alv}$), airborne concentrations of DMAc and DMAc retention ratio (R):

$$v = c_2 \cdot \dot{V}_{alv} \cdot R \tag{4.5}$$

wherein (c_2) is the concentration of DMAc in air.

Statistics

Inlet DMAc medium concentrations of every perfusion were calculated using perfusate DMAc concentrations, which were measured at fixed time intervals. Inlet DMAc medium concentrations were divided in five subgroups, mean extraction ratios and standard deviations were calculated. Differences in extraction ratios were tested using the Kruskal-Wallis and the Wilcoxon two-sample test. The statistical analyses were performed with SAS software.

Results

General effects

Table 4.1: Liver weight, bile production and LDH leakage in rat livers perfused at various concentrations of DMAc

n	C_0 (μM)	liver weight (g)	bile production ($mg \cdot min^{-1} \cdot g^{-1}$)	t_{LDH} (min)
3	0.0	10.8±1.8	1.03 (0.94, 1.11)	40±5
2	32.6 (31.8, 33.3)	8.3 (7.5, 9.0)	0.53 (0.38, 0.68)	38 (20, 55)
3	57.5±5.8	7.6±0.6	1.12±0.43	40±5
3	118.7±15.2	8.5±1.4	1.03±0.20	47±13
3	273.7±23.6	7.9±1.8	0.49 (0.32, 0.66)	43±3

C_0 is the DMAc medium concentration at the start of the perfusion. t_{LDH} is the time period after the start of the perfusion at which a LDH activity in the perfusate of 100 $U \cdot l^{-1}$ was reached.

The mean liver mass, weighed at the end of each perfusion, was 8.6 g (SD=1.7; n=14). Mean medium flow was 4.2 $ml \cdot min^{-1} \cdot g^{-1}$ (SD=0.4; n=14). When LDH concentrations were plotted against time, LDH leakage remained low during a period of about 40 minutes. Thereafter a steep rise in LDH leakage occurred. The 100 $U \cdot l^{-1}$ threshold gives a good measure for the length of the stable period (Table 4.1; see Palmen 1992 [15] for an illustration of this phenomenon). Addition of DMAc to the medium did not influence the results.

Mean bile production was 0.88 $mg \cdot min^{-1} \cdot g^{-1}$ (SD=0.36; n=12) and was not influenced by the addition of DMAc to the medium. Histological preparations of liver samples perfused with DMAc (haematoxilin-eosin staining), were normal in appearance (Figure 4.2).

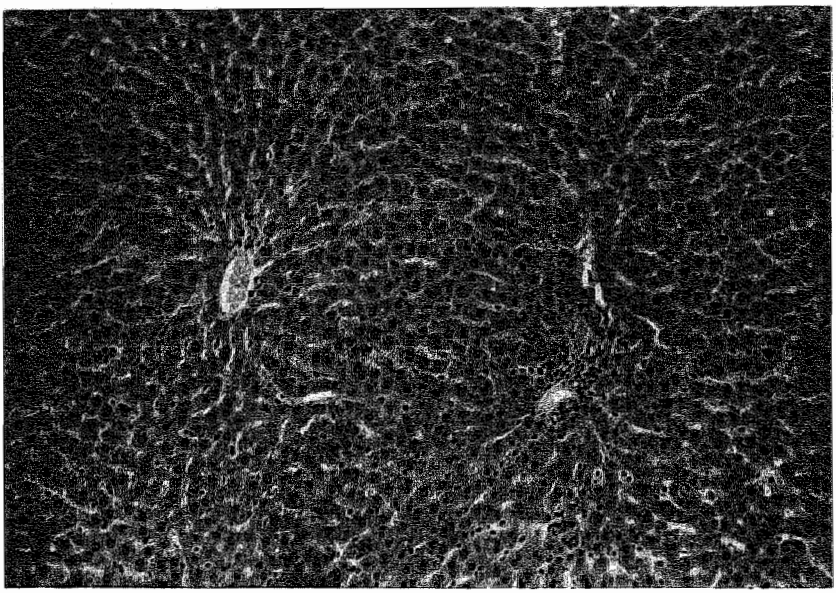

Figure 4.2: Histological evaluation of a liver perfused during a one hour recirculating perfusion after haematoxilin-eosin staining. Medium was composed of a bicarbonate buffered Krebs solution with 1 % albumin and glucose. 100 μM DMAc was added to the medium.

Rats pretreated with cimetidine or addition of cimetidine during the perfusion

The mean liver mass of livers pretreated with cimetidine was 9.7 g (8.3, 11.1; n=2) and 6.6 g (6.2, 6.9; n=2) when cimetidine was added during the perfusion. Mean medium flow was 3.8 $ml \cdot min^{-1} \cdot g^{-1}$ (3.2, 4.3; n=2) when rats were pretreated with cimetidine and 4.2 $ml \cdot min^{-1} \cdot g^{-1}$ (4.1, 4.2; n=2) when cimetidine was added to the medium (Table 4.2). The time period after the start of the perfusion at which a LDH activity of 100 $U \cdot l^{-1}$ was reached was 60 minutes or more when rats were pretreated with cimetidine and 30 minutes when cimetidine was added to the medium compared with 47 minutes when no cimetidine was used (Table 4.2). These differences were not statistically significant. Mean bile production was 0.91 $mg \cdot min^{-1} \cdot g^{-1}$ liver (0.82, 0.99; n=2) when rats were pretreated with cimetidine and 0.81 $mg \cdot min^{-1} \cdot g^{-1}$ liver (0.64, 0.97; n=2) when cimetidine was added to the medium, and was also not different within two standard deviations from perfusions without cimetidine.

Table 4.2: Effect of cimetidine on rat liver function during perfusion with DMAc.

perfusion type	n	C_0 (μM)	liver mass (g)	bile production $mg \cdot min^{-1} \cdot g^{-1}$	t_{LDH} (min)
A	3	118.7±15.2	8.5±1.4	1.03±0.20	47±13
B	2	101.9	9.7	0.91	>60
		(95.2, 108.6)	(8.3, 11.1)	(0.82, 0.99)	(>60, 60)
C	2	91.4	6.6	0.81	30
		(89.5, 93.4)	(6.2, 6.9)	(0.64, 0.97)	(30, 30)

Perfusion type: (A) no additions (B) cimetidine pretreatment (C) cimetidine added to the medium. C_0 (values are mean±SD) is the DMAc medium concentration at the start of the perfusion. t_{LDH} is the time period after the start of the perfusion at which a LDH activity in the perfusate of 100 $U \cdot l^{-1}$ was reached.

Determination of DMAc extraction ratios

Tissue DMAc concentrations were determined immediately after the cessation of the experiments and found to be negligible. The estimated volume of distribution for DMAc was 165 ml, indicating almost no protein binding, as 150 ml medium was used in the perfusion. DMAc inlet concentrations were calculated using perfusate DMAc concentrations and subsequently divided in five subgroups. Mean DMAc perfusate concentrations of these subgroups were 16 (n=29), 36 (n=50), 70 (n=34), 160 (n=18) and 225 (n=18) μM. Mean extraction ratios for the respective concentrations were 0.085, 0.061, 0.066, 0.047 and 0.044. DMAc extraction ratios were different at varying perfusate concentrations (Kruskal-Wallis; $p = 0.074$)(Figure 4.3).

The extraction ratio at a medium concentration of 16 μM was significantly higher than the extraction ratio at DMAc inlet concentrations of 36 μM, 70 μM, 160 μM or 225 μM (Figure 4.3). Extraction ratios did not differ significantly between groups with DMAc perfusate concentrations of 36 μM or more.

As cimetidine is reported to inhibit both the monooxygenase and oxidase activity of cytochrome P-450 [4, 18] the effect of cimetidine pretreatment or addition of cimetidine to the perfusion medium on DMAc extraction ratios was tested. As cimetidine perfusions were performed with starting DMAc concentrations of 100 μM, control perfusions with the same initial DMAc concentrations were selected. Inlet concentrations between 25 and 100 μM DMAc were compared between the groups (Figure 4.4).

Based on the results we may conclude that neither pretreatment of the rats with

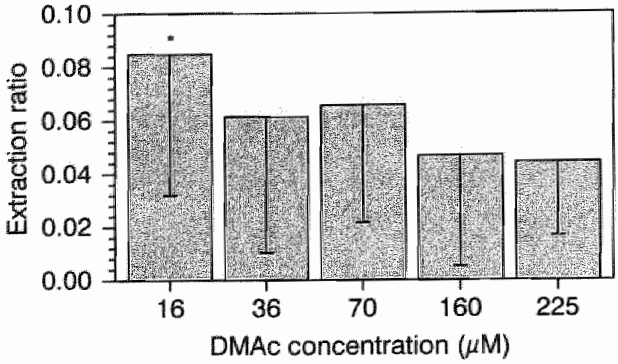

Figure 4.3: Mean dimethylacetamide (DMAc) extraction ratios plus standard deviations at varying DMAc inlet concentrations. The extraction ratio at a DMAc inlet concentration of 16 μM was significantly higher (Wilcoxon) compared to DMAc concentrations of 36 μM ($p = 0.039$), 70 μM ($p = 0.079$), 160 μM ($p = 0.007$) and 225 μM ($p = 0.010$), indicated by (*).

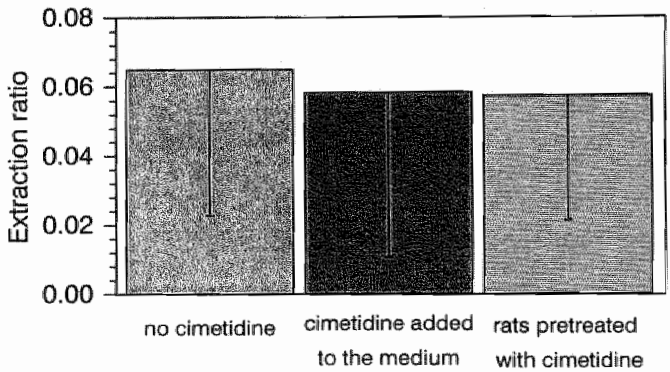

Figure 4.4: No effect of cimetidine addition to the perfusion medium or pretreatment of the rats with cimetidine on dimethylacetamide extraction ratio (SD). Inlet DMAc concentrations were between 25 and 100 μM.

cimetidine (75 mg·kg^{-1} rat) during three days before perfusion, nor addition of cimetidine to the perfusion medium 20 minutes after the start of the perfusion resulted in a decreased extraction of DMAc.

Calculation of airborne concentrations leading to saturation

The starting point for this calculation is the lowest perfusion concentration at which saturation of rat liver extraction was observed (36 μM; Figure 4.3). We

assumed that in humans saturation occurs at the same (plasma) level and used (extrapolated) rat data to calculate the airborne exposure at which such a plasma level is achieved.

Kinetic parameters in rat livers at the concentration where saturation occurs (36 μM):

mean rat liver weight	8.6 g
mean medium flow	4.2 ml·min^{-1}· g^{-1}
Extraction ratio	0.061
Clearance	2.20 ml·min^{-1}

Extrapolation of these findings to a human liver (1.5 kg) leads to a DMAc clearance of 380 ml·min^{-1} and a maximal metabolic rate of 14 μmoles·min^{-1}. The respiratory rate at modest physical exercise (50 W) is about 20 l·min^{-1}[1]. Assuming a retention ratio of 100 % an uptake equal to this metabolic rate would be reached at an airborne DMAc concentration of 18 ppm.

Discussion

To prevent its hepatotoxic activity at chronic exposure [5], the TLV for DMAc was set at 10 ppm (8 hr, TWA). As the concentration of MMAc, a metabolite of DMAc, in urine might be a good parameter for biological monitoring of DMAc exposure, a deeper understanding of kinetics and metabolism of DMAc is desirable. Particularly, when metabolism of DMAc is saturated, no further increase in urine MMAc concentrations at higher exposures can be expected. As previous data (Figure 4.1) suggest that DMAc metabolism might be saturated at its current TLV/TWA (10 ppm=35 mg·m^{-3}), we used the isolated perfused rat liver to gain an insight into DMAc kinetics and metabolism.

Our data show that the extraction ratio at a DMAc medium concentration of 16 μM (E=0.085; SD=0.053; n=29) was significantly higher than the extraction ratio at 36 μM DMAc (E=0.061; SD=0.051; n=50). The extraction ratios at DMAc medium concentrations of 36 μM or higher were not different from each other. Based on these findings we have concluded that DMAc metabolism was saturated at a DMAc medium concentration of 36 μM. As the detection limit of DMAc was 10 μM, it was not possible to test DMAc medium concentrations lower than 10 μM. For this reason, a DMAc medium concentration group below 16 μM could not be tested. Therefore, it can not be excluded that saturation already occurs at even lower concentrations.

Bile production and LDH leakage into the medium were not influenced by the addition of DMAc to the medium; histological examination of liver preparations perfused with DMAc (initial inlet concentration 100 μM) were normal, so liver impairment is not likely to be the cause of the lower extraction ratio.

As the analogue of DMAc, DMF, is N-demethylated by microsomal enzymes [2] and as both DMF and DMAc are demethylated, the two substances could possibly share the same metabolic pathway. Cimetidine, reported to inhibit N-demethylase activity of cytochrome P-450 [4,17], was tested with respect to its effect on DMAc extraction ratios. Neither cimetidine pretreatment nor addition of cimetidine to the medium had an effect on bile production or LDH activity. Also pretreatment of animals with cimetidine (75 $mg \cdot kg^{-1}$ per day) during the three days before the experiment, had no effect on microsomal DMAP metabolism or DMAc extraction ratio (Figure 4.4). The absence of an effect on DMAP N-demethylase activity indicates a recovery of this activity in microsomes after the final injection, given 10 hours before the preparation of the microsomes. Speeg *et al.* previously reported a full recovery of DMAP activity after 24 hours, but they did not check DMAP activity after shorter periods of time [18]. The absence of an effect of cimetidine addition on DMAc clearance in the perfused liver indicates that DMAc may be metabolised by other (iso)enzymes than DMAP.

Experimental error was low as the coefficient of variation of the analytical procedure for DMAc was 4 % for all DMAc concentrations above 10 μM. The large variation in the results shown in Figures 4.3 and 4.4 was caused by large inter-individual metabolic variation of the Wistar rats. As Wistar is an outbred strain, large inter-individual variation was to be expected. This variation in DMAc metabolism will probably lead to variation in the DMAc concentration at which saturation occurs. This knowledge might be of major concern regarding possible human variation in DMAc saturation. Variation in DMAc metabolism in humans was already suggested by Borm *et al.*, who found in two out of eight workers a significantly higher MMAc/DMAc ratio in urine [5].

The airborne DMAc concentration that would lead to a saturated metabolism in humans was based on the medium DMAc concentration at which saturation occurred in rat livers. Assuming modest physical exercise (50 W) with a respiratory ventilation of 20 $l \cdot min^{-1}$, it was calculated that saturation would occur at an air-borne DMAc concentration of 18 ppm. This implies that interpretation of data obtained for biological monitoring at values exceeding 25 $mg \cdot l^{-1}$ MMAc in urine, should proceed with utmost care (Figure 4.1).

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Chapter 5

Influence of oxygen supply on liver condition and elimination of dimethylacetamide in the isolated perfused rat liver

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Summary

The effects were studied of improved oxygen supply on the integrity and metabolic activity towards dimethylacetamide of the isolated perfused rat liver. Improvement of oxygen supply by increased medium oxygenation or addition of chemical oxygen carriers (perfluortributylamine) or erythrocytes led to increased bile secretion. Leakage of lactate dehydrogenase and aspartate aminotransferase could be prevented during a one hour perfusion when either chemical oxygen carriers or erythrocytes were added. Improved medium oxygenation alone was not sufficient to prevent high enzyme leakage during the second half of the perfusion period. Histological evaluation confirmed the conclusion that less damage occurred when erythrocytes or perfluortributylamine were added to the perfusion medium. The metabolic clearance of dimethylacetamide by the perfused rat liver was not significantly improved when erythrocytes were added to the medium. The results show that addition of perfluortributylamine, or erythrocytes at a level of 4 g haemoglobin·l^{-1}, is nescessary to maintain liver integrity for at least 1 hour in the liver perfusion system used in this study.

Introduction

Biotransformation and toxicity of xenobiotics can be studied by using test systems *in vitro*. The liver is the organ with the highest metabolic activity [14], and many test systems *in vitro* are based on this organ. In order of increasing conformity with the situation *in vivo* these test systems comprise preparations of (1) purified enzymes, (2) microsomes, (3) liver homogenates, (4) isolated hepatocytes, (5) liver slices and finally (6) the (isolated) perfused liver. Advantages of the perfused liver over the more basal systems are the intact cellular structure (which is also found in hepatocytes and liver slices) and the presence of all cell types in their normal cellular architecture. Medium flow can be kept constant and bile samples can be taken. Compared to the situation *in vivo*, liver metabolism can be studied without interference from metabolic processes in other organs; substances in concentrations that are not tolerated *in vivo* can be tested.

A standard liver perfusion technique does not exist. Variation between experimenters exists with regard to animal species, liver perfusion systems, composition of the medium, medium pressure and medium flow through the liver. For this reason it is difficult to compare liver condition between studies. In contrast to the conviction of many authors that medium without oxygen carriers keeps the liver intact for several hours, in a well-documented study Schmucker and Curtis had already reported in 1974 that this was not the case [18], even when the flow of medium through the liver was increased. However, when erythrocytes were added

to the perfusion medium, liver medium flow rates of 15 $ml \cdot min^{-1} \cdot g^{-1}$ liver were sufficient to maintain liver integrity. This is in agreement with the findings of Sies [19] who reported that oxygen saturation of the medium is a critical parameter during perfusion of the isolated liver. Early cell impairment may be a consequence of oxygen deficiency, which may affect kinetic and metabolic parameters. However, oxygen can also be toxic when its concentration in the medium is too high [5]. For use in the technique using the isolated perfused rat liver in the study of the metabolism of xenobiotics [12], we have studied the effects of various methods of increasing the oxygen supply to the liver. For this purpose, the addition of chemical oxygen carriers or erythrocytes to the medium, and changes in flow of the oxygenating medium were used. To our knowledge the effect of these medium conditions on liver integrity has not previously been tested in the same liver perfusion system in a single study. As liver medium flow rates of more than 3.5 $ml \cdot min^{-1} \cdot g^{-1}$ liver were damaging in their own right, we did not increase oxygen supply by increasing the flow rate of medium to the liver itself. Finally, kinetic parameters of dimethylacetamide were determined using both medium without addition of oxygen carriers and medium supplied with erythrocytes.

Materials and methods

The isolated perfused rat liver

Chemicals

Dimethylacetamide (DMAc), dimethylformamide and monomethylacetamide were supplied by Janssen Chimica (Beerse, Belgium), bovine serum albumin (BSA) by Organon Teknika (Boxtel, The Netherlands), Synperonic F68 by Serva (Heidelberg, Germany) and perfluortributylamine (FC43) by 3M Company (Leiden, The Netherlands). Lactate dehydrogenase (LDH; from rabbit muscle) EC 1.1.1.27 and malate dehydrogenase (from pig heart) EC 1.1.1.37 were obtained from Boehringer (Mannheim, Germany). Water was demineralized and filtered before use. All other chemicals were of analytical grade quality.

Animals

Male Wistar rats (Bor: WISW (SPFCpb)) weighing 250–280 g were used; they were deprived of food (SRMA 1210, Hope Farms) 12 hours before the start of the experiment, but had free access to water (pH 2.5). The rats were housed at 21°C, at a relative humidity of 55 %, and were kept on a night/day cycle from 07.00 until 19.00 hour.

Erythrocytes

Blood derived from human volunteers was obtained from the Red Cross Blood Bank Zuid Limburg, collected in vacuum vessels in ethylenediaminetetraacetic acid and stored overnight at 4°C.

Liver perfusion technique

The liver perfusion and all surgical procedures were done according to Meijer *et al.* [11]. Medium flow was kept constant during perfusion and regulated by changing hydrostatic pressure. Bile and perfusate samples were taken at regular time intervals. At the end of the perfusion, 1 hour after the start of a perfusion, a liver sample was taken for histological examination.

Perfusion medium

Standard medium was composed of a bicarbonate buffered Krebs solution with 1 % BSA and 11 mM glucose and was filtered (0.2 μm). For erythrocyte-amended medium, the erythrocytes were washed three times in 0.9 % NaCl and diluted in standard medium to a haemoglobin concentration of 4 $g \cdot l^{-1}$. In metabolic experiments, DMAc was added to the medium before the addition of erythrocytes. Medium amended with perfluortributylamine was prepared diluting 5.6 g of the detergent Synperonic F68 in deionized water (150 ml). After addition of 24 ml perfluortributylamine (FC43), the mixture was sonicated for 1 hour (40–50 W, 0–4°C) under carbon dioxide. 160 ml of this suspension was added to Krebs buffer (20 times concentrated), glucose and BSA to obtain the same component concentration as in standard medium. The perfluortributylamine medium was filtered (8 μm) before use.

Study design

Liver integrity using different medium conditions was tested during a one hour perfusion period. Oxygen supply to the liver was modified by manipulation of the medium flow rate *through the oxygenators* by way of an extra loop in medium flow, or by addition of a chemical oxygen carrier (perfluortributylamine) or erythrocytes to the medium. *Liver* medium flow was 3.5 $ml \cdot min^{-1} \cdot g^{-1}$ liver for every perfusion performed. The effect of a high flow rate of medium through the artificial lungs (186 $ml \cdot min^{-1}$; n=3) compared with a low flow rate of medium (92 $ml \cdot min^{-1}$; n=5) in standard medium was determined. Perfluortributylamine-supplemented medium (n=2) in perfusions with a low flow rate of medium, and erythrocytes added to perfusions with a high flow rate of medium (n=4) were also evaluated. Bile production, LDH and aspartate aminotransferase (ASAT; EC 2.6.1.1) activity in perfusate samples were determined and histological preparations were made.

The biotransformation capacity of the liver was studied by addition of DMAc (90 and 120 μM) to a perfusion medium with and without erythrocytes. A plot of logarithmic concentration v. time was used to determine the elimination rate constants (k). Corrections were made for the removal of medium during sampling. Clearance was calculated by multiplying k by the volume of distribution. The extraction ratio was calculated by division of clearance by the actual medium flow.

Analytical procedures

Bile samples were taken at regular intervals. Bile secreted during perfusion was expressed as mass per minute per gram liver. LDH activity of the perfusate samples was determined according to Kreutzer (1989), ASAT activity according to Moss (1986). Albumin was added to the perfusate samples (final concentration 3 %) and stored at -80°C until determination. Histological preparations (haematoxilin-eosin staining) of the liver were made immediately after cessation of the experiment and examined by light microscopy.

Gas chromatochraphic analysis

Samples (1 ml) were saturated with sodium chloride and extracted with an equal volume of acetonitrile. After addition of an internal standard (dimethylformamide, final concentration 36 μM), 0.2 μl of the extracted sample was analyzed for DMAc and its metabolite monomethylacetamide by gas chromatography. A Carlo Erba 5300MS (Interscience) gas chromatograph fitted with a DB-WAX 20M column (0.326 mm$\times$30 m, film thickness 0.5 μm) and an on-column injection unit was used. Column temperature was 120°C (isothermal), and the detector (flame ionization detector) temperature was set at 240°C. Helium was used as a carrier gas at a flow rate of 3 ml$\cdot$min^{-1}. Under these conditions the retention times for DMAc and monomethylacetamide were 5.2 and 12.2 minutes, respectively. Peak-height ratios of DMAc and dimethylformamide were determined and DMAc concentrations in the perfusate samples were calculated. Under these conditions the detection limits were 10 and 35 μM for DMAc and monomethylacetamide respectively. Final concentrations in the perfusate were corrected for the decrease in medium volume.

Results

Viability of the isolated perfused rat liver

Bile secretion

Table 5.1: Mean bile secretion of individual perfusions

Additions to the standard medium	oxygenator medium flow rate ($ml \cdot min^{-1}$)	mean bile secretion ±SD for individual perfusions ($mg \cdot min^{-1} \cdot g^{-1}$ liver)
None	92	0.59±0.28
		0.26±0.21
		0.77±0.37
		0.28±0.11
		0.44±0.23
None	186	0.18±0.03
		0.97±0.23
		0.94±0.09
FC43	92	0.80±0.48
		0.58±0.30
Erythrocytes	186	1.33±0.17
		1.39±0.29
		1.16±0.16
		0.56±0.06

Bile secretion of individual perfusions with low and high flow rates over the oxygenators and with and without addition of perfluortributylamine (FC43) or erythrocytes. Mean±standard deviations within the individual perfusions are given.

Bile secretion varied significantly between individual perfusions, even under the same conditions (Table 5.1). The influence of medium conditions on bile secretion is illustrated in Figure 5.1. Data from individual perfusions were standardized with respect to the mean value for the respective medium condition 45 minutes after the start of the perfusion. In all cases, bile production was low directly after the onset of the perfusion and increased to a steady level, which was attained after about 20 minutes. Bile secretion under low oxygen conditions was relatively low (± 0.4 $mg \cdot min^{-1} \cdot g^{-1}$ liver). Both an increase in oxygen saturation of medium and the addition of standard chemical oxygen cariers resulted in increased bile production (± 0.6 $mg \cdot min^{-1} \cdot g^{-1}$ liver). When erythrocytes were added to the medium at a high flow rate through the oxygenators, the bile secretion was even

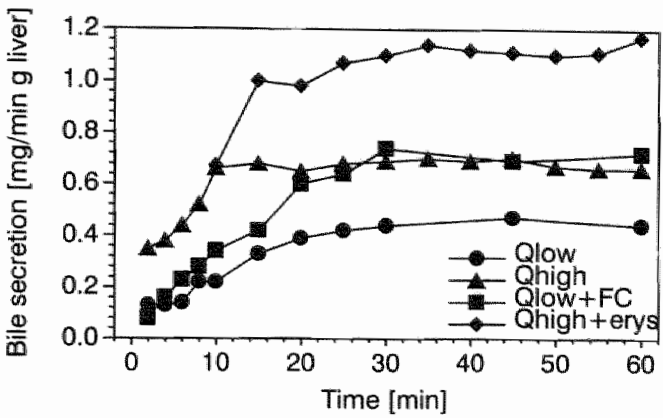

Figure 5.1: Time course of bile secretion during perfusions with standard medium and an oxygenator medium flow of 92 ml·min^{-1} (Q_{low}; n=5); the same medium composition but an oxygenator medium flow of 186 ml·min^{-1} (Q_{high}; n=3); perfluortributylamine (FC43) added to the standard medium with an oxygenator medium flow of 92 ml·min^{-1} (Q_{low} + FC; n=2); erythrocytes (Hb=4 g·l^{-1}) added to the standard medium and an oxygenator medium flow of 186 ml·min^{-1} (Q_{high} + erys; n=4). The values are means of all perfusions performed in that category.

higher (± 1.1 mg·min^{-1}· g^{-1} liver).

Lactate dehydrogenase and aspartate aminotransferase release

Enzyme leakage during the first 30 minutes after the start of the perfusion was low in all cases. Thereafter, the leakage of LDH and ASAT increased progressively in the absence of oxygen carriers (Figure 5.2). Increased flow rate of the medium through the artificial lungs did not influence enzyme leakage. When perfluortributylamine or erythrocytes were added to the medium, the enzyme leakage remained low during the whole perfusion period. A LDH perfusate activity of 300 U·l^{-1} and an ASAT activity of 75 U·l^{-1} were reached in the perfusate after about 45 minutes, when no oxygen carriers were added to the medium; both activities increased markedly thereafter. When chemical oxygen carriers or erythrocytes were added to the medium, these enzyme activities were not reached within a one hour perfusion period.

Histological evaluation

Histological evaluation was in agreement with data obtained from enzyme leakage determination. Vacuolization and necrosis in the central lobular area (Rappaport areas 2 and 3) occurred when no chemical oxygen carriers or erythrocytes were

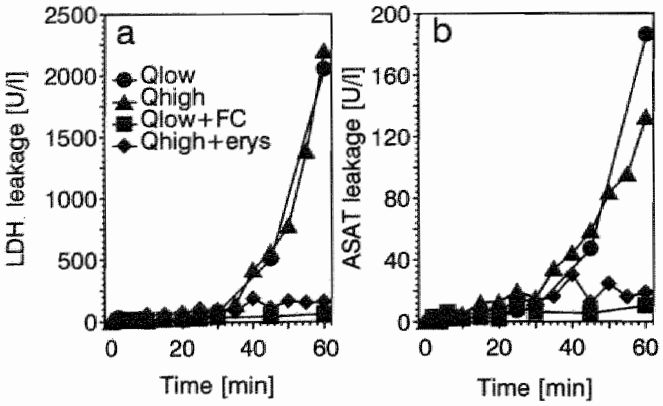

Figure 5.2: Time course of (a) lactate dehydrogenase (LDH) and (b) aspartate aminotransferase (ASAT) activity during perfusions with standard medium and an oxygenator medium flow of 92 ml·min^{-1} (Q_{low}; n=5); the same medium composition but an oxygenator medium flow of 186 ml·min^{-1} (Q_{high}; n=3); perfluortributylamine (FC43) added to the standard medium with an oxygenator medium flow of 92 ml·min^{-1} (Q_{low} + FC; n=2); erythrocytes (Hb=4 g·l^{-1}) added to the standard medium and an oxygenator medium flow of 186 ml·min^{-1} (Q_{high} + erys; n=4). The values are means of all perfusions performed in that category.

used in perfusions with a low and a high flow rate of medium through the oxygenator (Figure 5.3). Addition of perfluortributylamine or erythrocytes to the medium appeared to prevent hepatocellular damage caused by the perfusion; however, sinusoids were more dilated using perfluortributylamine (Figure 5.4).

Metabolic activity

DMAc, which was used as a substrate to study biotransformation activity of the liver, was added to the medium just before the start of the perfusion. After perfusion was started, DMAc disappeared from the medium with a first-order 'elimination' rate constant of 0.019 ± 0.008 min^{-1} (n=3) without and 0.022 ± 0.014 min^{-1} (n=4) with addition of erythrocytes (difference not statistically significant by Wilcoxon two sample test; Figure 5.5). The disappearance of DMAc from the medium was clearly 'liver dependent' as the DMAc concentration remained constant during 'perfusions' without livers, regardless of the presence of erythrocytes. Monomethylacetamide, a metabolite of DMAc [2, 6] could not be detected in perfusate samples (less than 35 μM).

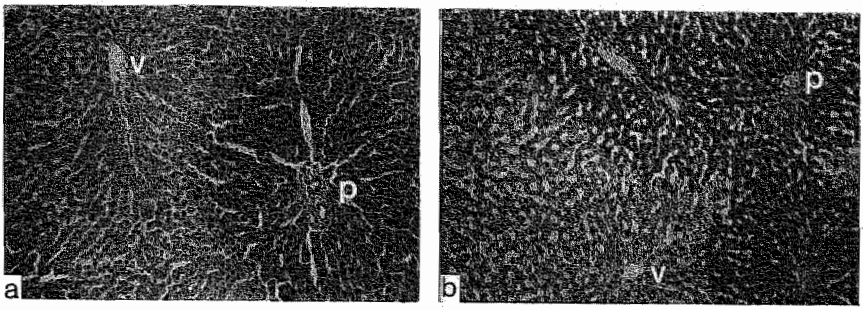

Figure 5.3: Histological evaluation of livers perfused with a 1-hr recirculating perfusion after haematoxilin-eosin staining. Medium was composed of standard medium and (a) an oxygenator medium flow of 92 ml·min^{-1} or (b) 186 ml·min^{-1}. P = Afferent terminal (portal) venule; V = efferent terminal (hepatic) venule. Note the extensive necrosis in the central lobular areas (Rappaport areas 2 and 3). Original magnification×160.

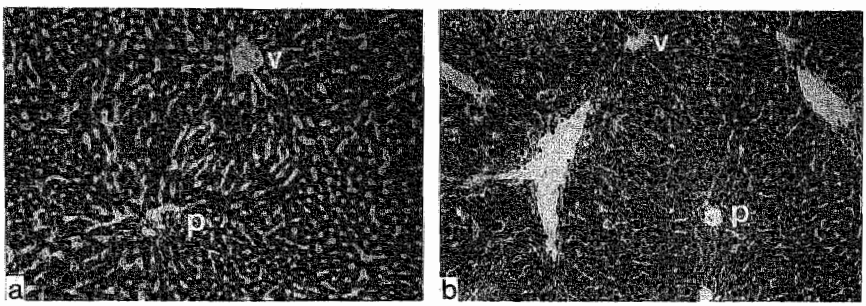

Figure 5.4: Histological evaluation of livers perfused with a 1-hr recirculating perfusion after haematoxilin-eosin staining. Medium was composed of standard medium and (a) perfluortributylamine (FC43) with an oxygenator medium flow of 92 ml·min^{-1} or (b) erythrocytes (Hb=4 g·l^{-1}), and an oxygenator medium flow of 186 ml·min^{-1}. P = Afferent terminal (portal) venule; V = efferent terminal (hepatic) venule. Note the dilated sinusoids in the FC43-perfused liver and the healthy appearance of the erythrocyte-perfused liver. Original magnification×160.

Discussion

The isolated perfused rat liver technique is often used to study the metabolism of xenobiotics because the metabolism is not influenced by external, hormonal or neural factors, and metabolic activity of other organs than the liver is ex-

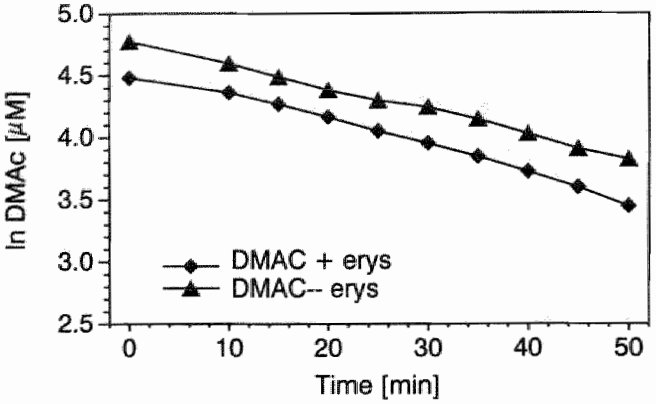

Figure 5.5: Time course of dimethylacetamide (DMAc) concentration during perfusions with standard medium and an oxygenator medium flow of 186 ml·min^{-1} (DMAc-erys; ▲, n=3), or perfusions with the same medium supplied with erythrocytes (Hb=4 g·l^{-1}) and an oxygenator medium flow of 186 ml·min^{-1} (DMAc+erys; ♦, n=4). DMAc concentrations were means of all perfusions performed in that category.

cluded. The type and quantity of metabolites formed is influenced substantially by the composition of perfusate, degree of oxygenation, temperature, perfusate flow rate, concentration of the substrate tested and the presence of inhibitors or inducers [14]. Medium composition is often rather artificial compared with that of normal blood, which may have a considerable influence on the metabolic activities of the liver [10]. Inadequate oxygenation of the medium may reduce substrate extraction, especially for downstream hepatocytes [15]. Thurman and Scholz [20] reported that oxygen uptake in perfused livers from normal and phenobarbital-treated rats was 100–125 μmoles·g^{-1}·h^{-1}. Using phenobarbital-pretreated starved rats, oxygen consumption of the liver was found to increase by 16 μmoles·g^{-1}·h^{-1} when the monooxygenase substrate aminopyrine was added to the medium.

Bile formation, as an indicator of the functionality of the secretory mechanisms, is often used as a parameter to evaluate liver integrity. Normal bile secretion rate is of the order of 0.9 mg·min^{-1}· g^{-1} [7] and is impaired by hepatotoxic compounds such as chloroform [4]. Bile formation requires the expenditure of metabolic energy for the performance of osmotic work, and depends on many factors such as perfusate flow rate and temperature [3]. In the present study the low rate of bile production at the beginning of the perfusions can be explained by sub-optimal conditions (e.g. medium temperature) caused by technical adjustments. Bile production rate in our perfusions with standard medium at high flow rate through the artificial lungs was about equal to that reported by Krell *et al.* [7]. When standard medium at a low flow rate over the oxygenators was used, the amount

of bile secreted was only about half this value. The addition of oxygen carriers increased bile production. The flow rate through the liver did not vary between experiments.

ASAT activities after one hour perfusions using medium containing diluted rat blood were reported to be in the range of 60–80 $U \cdot l^{-1}$ [1,9]. In the present study ASAT activity was about 10 $U \cdot l^{-1}$ after a 1 hour perfusion with medium containing erythrocytes or perfluortributylamine, indicating that cell membranes were still intact. Without these additions much higher ASAT leakage was found (Figure 5.2).

Histological examination of the liver after perfusions with erythrocytes or perfluortributylamine revealed less impairment than after perfusions without additions. These results were in agreement with those of Schmucker and Curtis [18] who reported extensive injury of the sinusoidal endothelium and vacuolization in centrilobular parenchymal cells caused by oxygen deficiency using standard medium. Increasing the flow through the liver from 15 to 72 $ml \cdot min^{-1}$ in the erythrocyte-free system still resulted in injured centrilobular cells; addition of erythrocytes to the Krebs medium solved this problem. Perfluortributylamine caused 'ballooning' of sinusoids; this phenomenon was also reported by Pang [14].

Oxygen supply is not only important to maintain liver function, but also for metabolic activity. As the pericentral oxygen concentration is lower than the periportal oxygen concentration, and oxidative reactions of substrates requiring oxygen are often mediated by the cytochrome P-450 monooxygenation system, which is concentrated in downstream hepatocytes [15], metabolism of substrates could be impaired when oxygen supply is deficient. Theophylline, for example, is catabolized chiefly by oxygen-dependent enzymes [12]; when the oxygen tension in the medium decreased, theophylline clearance was also found to be decreased. In spite of a better oxygen supply to the liver when erythrocytes were used, no significant differences in DMAc metabolism were found in the present study between erythrocyte-amended medium (n=4) and standard medium (n=3). The extraction ratio for DMAc was 0.0579 ± 0.030 when no erythrocytes were added, and 0.073 ± 0.041 when erythrocytes were added to the medium.

On the basis of our results we may conclude that oxygen supply to the liver is critical for liver function. Standard medium and a low flow rate of medium through the oxygenator (92 $ml \cdot min^{-1}$) leads to impaired bile secretion, increased enzyme activity in the perfusate and microscopically visible damage. When the flow rate of medium through the oxygenator was increased (186 $ml \cdot min^{-1}$), bile secretion increased but perfusate enzyme activity remained high and microscopy still showed heavily damaged cells in the central lobular region. Both bile secretion and perfusate enzyme activity were closer to normal when oxygen carriers were used. Fluorocarbons (e.g. perfluortributylamine) may act as monooxygenation uncouplers [21] and are phagocytized by Kupffer cells [17], a process that

was reported to cause depression of cytochrome P-450 levels and a decrease in the related drug biotransformation [16]. In our opinion, therefore, the use of perfluortributylamine is not advisable in biotransformation studies. In conclusion, erythrocytes added to standard medium lead to good bile production and low perfusate enzyme activities. Elimination of DMAc, however, was not significantly improved using medium supplied with erythrocytes.

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Chapter 6

Oxidative effects in human erythrocytes caused by some oximes and hydroxylamine

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Submitted for publication

Summary

Both oximes and hydroxylamine (HYAM) are compounds with known oxidative capacity. We tested *in vitro* whether acetaldoxime (AAO), cyclohexanone oxime (CHO), methyl-ethyl ketoxime (MEKO) or hydroxylamine (HYAM) affect hemoglobin oxidation (into $HbFe^{3+}$), thiobarbituric acid reactive substances (TBARS) formation, and glutathione (GT) depletion in human hemolysate, erythrocytes or blood. All these parameters are known to be related to oxidative stress. Glutathione S-transferase (GST) activity was measured as it may be affected by oxygen radicals. All three oximes caused a low degree of $HbFe^{3+}$ accumulation in erythrocytes. This was higher in hemolysates indicating that membrane transport may be limiting. $HbFe^{3+}$ accumulation for the oximes was lower than for HYAM. AAO and HYAM caused TBARS formation in blood. For HYAM this was expected as free radicals are known to be generated during $HbFe^{3+}$ formation. Free radical generation by AAO and HYAM in erythrocytes was confirmed by the inhibition of GST. For the other two oximes (CHO and MEKO) some special effects were found. CHO did inhibit erythrocyte GST while it did not cause TBARS formation. MEKO was the least potent oxime as it caused no TBARS formation, little $HbFe^{3+}$ accumulation and little GST inhibition in erythrocytes. However, GT depletion was more pronounced than for the other oximes, indicating that glutathione conjugation occurs. TBARS formation, GT depletion and GST modulation caused by the oximes and HYAM were also tested in rat hepatocytes. However, no effects were found in hepatocytes. This suggests that a factor present in erythrocytes is necessary for free radical formation.
Studies with suggested metabolites of the oximes (i.e. cyclohexanone, acetaldehyde or methyl-ethyl ketone) and addition of rat liver preparations to the erythrocyte incubations with oximes, suggest that metabolism is not a limiting factor in erythrotoxicity.

Introduction

Oximes are compounds containing the common structure HO–N=C and are therefore structurally related to hydroxylamine (HO–NH_2: HYAM). Among other applications, oximes are used in paints and enamels. Cyclohexanone oxime (CHO) is used in the production of caprolactam, which in turn is utilized in the production of nylon. Some oximes are known hematotoxic agents. Subacute and subchronic oral exposure of rats to CHO causes erythrocyte deformations (Howell Jolly bodies), hemoglobin oxidation, a decreased number of erythrocytes, a decrease in hemoglobin content and a lower hematocrit, with an accompanying increase in circulating reticulocytes and nucleated erythrocytes [13,21]. Chronic exposure of rats to methyl-ethyl ketoxime (MEKO) (subcutaneous injections) causes hypertrophy of the spleen, and a decrease in erythrocyte count and hemoglobin content [34]. These studies indicate that the primary target organ *in vivo* is the erythrocyte, causing hemolytic anemia with compensatory extramedullary

erythropoiesis, as well as hemosiderosis in the spleen. It is not well understood whether the hematotoxic effects associated with these oximes are due to the parent compound per se or to their metabolites. There are indications that oximes may be hydrolyzed to yield the corresponding aldehyde or ketone structure plus HYAM [28,42], a compound with strong oxidative capacity with regard to hemoglobin [23].

Due to their abundancy and presence all over the body, erythrocytes can easily become a target for toxic compounds. Under normal conditions, erythrocytes are exposed to oxidant stress caused by the high concentration of molecular oxygen and oxidizing substances released into the blood [22]. In order to offer resistance to these challenges, the erythrocyte contains large amounts of protein and non-protein anti-oxidants. When oxidant stress increases to abnormal levels, damage may occur. Xenobiotics may also increase the oxidant stress by disturbing the anti-oxidant systems, the erythrocyte membrane- or hemoglobin structures [22,51]. Normal physiological (auto)oxidation of hemoglobin is known to lead to superoxide formation [8]. Recently Stolze and Nohl [49] demonstrated liberation of other free radical products as a result of hemoglobin oxidation by hydroxylamine. Since the oxidation of hemoglobin to methemoglobin is a one electron step, radical formation is expected as a general result. This may initiate lipid peroxidation of the erythrocyte membrane, which can be measured as thiobarbituric acid reactive substances (TBARS) [2]. Free radicals can also oxidise protein and non-protein thiols. Glutathione is the most important non-protein thiol and may be oxidized into GSSG [12]. Peroxidized lipid products may be reduced by glutathione peroxidase or glutathione S-transferase (GST) at the expense of reduced glutathione generating GSSG [18]. When the reductive capacity of the cell is insufficient, GSSG can be released from the cell [12,33]. In addition to excretion of GSSG, the erythrocyte glutathione content may also be depleted by mixed disulfide formation [24]. Erythrocyte GST is a GST-π class enzyme [5], which is known to be inactivated by free radicals [14,15,16,38,45,46].

The aim of the present study was to test whether the oximes acetaldoxime (AAO), CHO and MEKO as such are able to generate oxidative effects like hemoglobin oxidation, TBARS formation and glutathione depletion or GST activity changes in human erythrocytes.To study whether metabolites of the oximes are able to generate these effects, two distinct approaches were used. On one hand, AA, CH, MEK and HYAM, which are suggested metabolites of AAO, CHO and MEKO were tested. On the other hand, oximes were also tested in a combined assay of erythrocytes and microsomes or hepatocytes, and in an isolated rat liver perfusion in which human erythrocytes were added to the medium. We tested whether metabolites generated by these metabolizing systems were able to generate oxidative effects in erythrocytes and/or hepatocytes.

Materials and Methods

Chemicals

Hydroxylamine (HYAM (ACS reagent); 99.1 %; CAS: 5470-11-1) and all non-specified enzymes and cofactors were from Sigma (St. Louis, USA), cyclohexanone (CH; >99.5 %; CAS: 108-94-1) and methyl-ethyl ketone (MEK; >99.5 %; CAS: 78-93-3) were from Fluka Chemie (Buchs, Switserland), acetaldehyde (AA; >99 %; CAS: 1632-89-9) was from Merck (Schuchardt, Germany). Cyclohexanone oxime (CHO; >99.5 %; CAS: 100-64-1), methyl-ethyl ketoxime (MEKO; >99 %; CAS: 96-29-7) and acetaldoxime (AAO; 99.7 %; CAS: 107-29-9) were a gift from DSM special products (Geleen, The Netherlands). Collagenase B (EC 3.4.24.3; from Clostridium histolyticum; Lot: 12086224-05) was obtained from Boehringer (Mannheim, Germany). Water was demineralized and microfiltrated before use. All other chemicals were of analytical grade quality.

Preparation of erythrocytes and lysate

Human blood was obtained from the Red Cross Blood Bank "Zuid Limburg" where it had been collected in sterile vacuum EDTA vessels and kept overnight at 4°C. Erythrocytes from at least 4 persons were pooled, washed three times using saline and diluted in Krebs-Henseleit buffer (pH 7.4) to a hemoglobin (Hb) concentration of 27 $g \cdot l^{-1}$ in incubations with transwells^{TM} (Costar, Cambridge) or washed erythrocytes, and 23 $g \cdot l^{-1}$ in incubations with microsomes. For isolated liver perfusions, erythrocytes were diluted in Krebs-Ringer buffer solution (pH 7.4) to a Hb concentration of 4 $g \cdot l^{-1}$ [40]. Hb concentrations were determined according to van Kampen and Zijlstra [31]. Lysate was prepared from packed erythrocytes by dilution with ice-cold water (1:2).

Preparation of microsomes and isolation of hepatocytes

Male Brown Norway rats (BN/M) of approximately 250 g were used. Housing was as previously described [40]. Animals were anesthetized with pentobarbital (Narcovet^{TM}; 0.1 ml/100 g rat). Microsomes were prepared as described previously [41] Microsomal cytochrome P-450 content was determined in duplicate using the dithionite-difference method according to Rutten *et al.* [43], and was found to be 0.78–0.85 nanomoles$\cdot mg^{-1}$ protein. The procedure for the isolation of hepatocytes was based on the methods described by Berry and Friend [3] and by Seglen [44]. A calcium and magnesium free buffer solution (100 mM NaCl, 7 mM KCl, 39 mM HEPES and 15 mM NaH_2PO_4 (pH adjusted to 7.4 with NaOH)) was perfused (30 $ml \cdot min^{-1}$) in a single pass way through the isolated rat liver during 20 minutes. Next, a collagenase containing buffer (0.05 $g \cdot 100\ ml^{-1}$), composed of 67 mM NaCl, 7 mM KCl, 100 mM HEPES and 5 mM $CaCl_2 \cdot 2H_2O$ (pH adjusted to 7.4 with NaOH), was perfused in a recirculating way during 5 minutes.

Hepatocytes were isolated from the liver, filtrated (100 μM) and washed three times in a Krebs-Henseleit buffer (pH 7.4; 4 minutes, centrifuge force: 25×g). The viability of the cells (over 85 %) was determined with the Trypan-blue exclusion test [29].

Incubations

Incubations with whole blood or hemolysate

Both in incubations with whole blood and in incubations with lysate (final volume 3.5 ml), 350 μl test compound (i.e. CHO, AAO, MEKO, HYAM, CH, AA and MEK), diluted in Krebs-Henseleit buffer or in water respectively (final concentrations between 1 and 7 mM), was added and incubated for 1 hour at 37°C in a shaking water bath (80 revolutions per minute). In hemolysate, $HbFe^{3+}$ accumulation, TBARS formation, activity changes of GST, and depletion of the sum of both reduced and oxidized glutathione (GT) were measured to test CHO, AAO, MEKO, HYAM, CH, AA and MEK. In blood, TBARS formation was tested for all compounds; $HbFe^{3+}$ accumulation, GST activity changes and GT depletion were tested only for CH, AA or MEK. Each day the incubations with whole blood or hemolysate were performed in triplicate. The experiments were performed three times on different days.

Incubations with erythrocytes

The incubations of erythrocytes were performed similar to the co-incubations of erythrocytes and hepatocytes (see below), replacing hepatocytes by Krebs-Henseleit buffer. CHO, AAO and MEKO (final concentrations 1, 2 and 3 mM) and HYAM (final concentration 3 mM) were tested. In erythrocytes $HbFe^{3+}$ accumulation, GST activity changes and GT depletion caused by CHO, AAO, MEKO or HYAM were measured. The incubations were tested in duplicate each day and were performed three times on different days.

Co-incubation of erythrocytes and microsomes

Incubations using washed erythrocytes (Hb 23 $g \cdot l^{-1}$) plus microsomes were performed as described previously [41]. The erythrocytes were washed three times with saline after an incubation period of 1 hour. AAO and MEKO concentrations between 1 and 7 mM, and CHO concentrations of 3.1 and 7.8 mM were tested. $HbFe^{3+}$ accumulation, GST activity changes and GT depletion were measured. Each day the incubations were performed in triplicate. The experiments were performed twice on different days.

Incubations with hepatocytes

Hepatocytes were diluted to a cell concentration of 6.6×10^6 cells·ml^{-1} with Krebs-Henseleit buffer. Hepatocytes (1 ml) were incubated in triplicate with CHO, AAO, MEKO or HYAM (0.5 ml, final concentration between 1 and 7 mM) or CH, AA or MEK (0.5 ml, final concentration 7 mM), for 1 hour at 37°C in a shaking water bath (80 revolutions per minute). GST activity changes, GT depletion and TBARS formation were measured. In each experiment all incubations were performed in triplicate. The experiments were performed three times on different days.

Co-incubation of erythrocytes and hepatocytes

Co-incubations of erythrocytes and hepatocytes were performed in transwells (a well in which a transwell, containing a porous 0.4 μm membrane, can be placed (Costar, Cambridge, England)) Erythrocytes (2.6 ml, Hb 27 g·l^{-1}) were pipetted into the well, and the hepatocytes (1 ml, 6.6×10^6 cells·ml^{-1}) plus CHO, AAO or MEKO (final concentrations between 1 and 3 mM) or HYAM (final concentration 3 mM) were added to the transwell. $HbFe^{3+}$ accumulation, GST activity changes and GT depletion were measured. Each day the experiment was performed in duplicate and repeated three times on different days.

To optimize cellular contact between hepatocytes and erythrocytes, co-incubations were also performed in which the two cell types were mixed. Cellular volume and concentrations were identical to those of the transwells. CHO, AAO, MEKO or HYAM concentrations were between 1 and 3 mM. After a one hour incubation period, erythrocytes were separated from the hepatocytes by density centrifugation using isotonic Percoll (gravity 1.08 g·l^{-1}, 4°C, centrifuge force 400×g). Erythrocytes were sampled and washed three times with saline (15 mM). In a single experiment, each test concentration was tested in triplicate.

Isolated rat liver perfusions

Liver perfusions were performed as described previously [40]. Medium was composed of Krebs-Ringer buffer solution (pH 7.4) containing washed human erythrocytes (Hb concentration 4 g·l^{-1} plus 0.1 % bovine serum albumin to prevent cell lysis). CHO, AAO or MEKO (final concentration 3 mM) were added to the perfusion medium before the start of the perfusion (n=1). In addition, liver perfusions without test compound were performed (n=2). Medium flow was kept constant for the perfusion. Bile samples were taken every 10 minutes. Mean bile production was 0.6 (SE=0.1) mg·min^{-1}· g^{-1} liver. Perfusate samples were taken before the start of the perfusion and every 10 minutes thereafter during 1 hour. $HbFe^{3+}$ accumulation, GST activity changes and GT depletion were measured. Liver damage was assessed through lactate dehydrogenase (LDH) activity (EC 1.1.1.27) in the

perfusate samples, using NADH consumption during pyruvate transformation to lactate [37]. Albumin was added to these samples prior to storage (-70°C; final concentration 3 %). Mean lactate dehydrogenase leakage was never higher than 100 $U \cdot l^{-1}$. Bile production and lactate dehydrogenase leakage indicate that liver viability was good [40].

Analytical procedures

Percentage of methemoglobin ($HbFe^{3+}$)

Samples were prepared according to the following procedures: a) In incubations with whole blood, 500 μl blood was centrifuged (2000×g, 5 min.). After removing the plasma, erythrocytes were lysed with 2 ml ice cold water and kept on ice during 15 minutes. b) Washed erythrocytes (1.2 ml, Hb 27 $g \cdot l^{-1}$ or 23 $g \cdot l^{-1}$) were concentrated by centrifugation (2000×g, 5 min.). After removing the supernatant, the erythrocytes were lysed with 2.25 ml ice cold water and kept on ice during 15 minutes. Perfusate samples derived from liver perfusions (2.7 ml, Hb 4 $g \cdot l^{-1}$) were also centrifuged (2000×g, 5 min.). After removing the supernatant, the erythrocytes were lysed with 750 μl ice cold water. c) Lysate (750 μl) was diluted with 1.5 ml water.

After the addition of potassium phosphate buffer (150 mM, pH 6.6, 1:1 v/v) to the samples, the cellular debris was removed by centrifugation (2000×g, 3 min.). The percentage $HbFe^{3+}$ present in hemolysates was calculated from the absorbance change after addition of KCN compared to the same change in a sample fully converted to the $HbFe^{3+}$ form by the addition of $K_3Fe(CN)_6$ as described by Betke *et al.* [4].

Thiobarbituric acid reactive substances (TBARS)

Cells were removed from the incubations by centrifugation (2000×g for blood cells and 25×g for hepatocytes, 5 min.). TCA (10 %) was added to the supernatants (1:1 v/v). After centrifugation of the denaturated samples (2000×g, 5 min.), the supernatant was incubated with thiobarbituric acid (TBA; 1 %, 1:1 v/v) during 15 minutes (100°C) and cooled immediately thereafter (10°C). The malondialdehyde-thiobarbituric acid complex was extracted into 1-butanol and the difference in absorption between the measurements at λ=535 nm and λ=590 nm was determined. TBARS concentrations were calculated using a molar extinction coefficient of 1.56×10^5 $l \cdot mol^{-1} cm^{-1}$ [47,48]. As AAO and AA also react with TBA, incubations with these compounds were corrected for this back-ground reactivity.

Glutathione concentrations

Samples derived from whole blood, hemolysate or hepatocytes were denaturated with TCA (8 %, 1:1 v/v) and centrifuged (10000×g, 5 min). The supernatant was diluted with 100 mM sodium/potassium phosphate buffer (pH 7.4, 1:8 v/v) and stored (-70°C) until determination. Erythrocytes from the erythrocyte incubations (150 μl) and from the perfusion experiments (400 μl) were concentrated by centrifugation. 25 μl TCA (8 %) was added to the pellet and the samples were frozen until determination (-70°C). After thawing, the samples were diluted 50 and 25 times respectively, with sodium/potassium phosphate buffer (100 mM), and centrifuged (10000×g, 3 min). GT was determined with the cyclic oxidation reduction method essentially as described by Anderson [1].

Glutathione S-transferase (GST) activity

Blood cells and hepatocytes were lysed (15 min.) by addition of four volumes ice cold water containing 1.4 mM dithiothreitol (DTT). Erythrocytes from the purified erythrocyte and co-incubation experiments were first concentrated by centrifugation (2000×g, 5 min.) of 1 ml sample. The pellets were diluted with 125 μl potassium phosphate buffer (pH 6.5) so that Hb concentrations of these samples were comparable with the samples derived from whole blood. Erythrocytes in perfusate samples from liver perfusions (Hb about 4 $g \cdot l^{-1}$) were concentrated by centrifugation (2000×g, 5 min.) of 1.3 ml sample. Ice cold water (450 μl) containing 1.4 mM DTT was pipetted on the erythrocyte pellet in order to lyse the erythrocytes. Ion strength of hepatocyte, whole blood and erythrocyte samples were re-established by addition of an equal volume of two fold concentrated phosphate buffered saline containing 1.4 mM DTT. In incubations with lysate, the same procedure was used with concentrated PBS containing 2.8 mM DTT.

Samples were centrifuged (10000×g, 5 min) and the supernatant was stored until determination (-70°C). After thawing the samples on ice, the GST activity towards chlorodinitrobenzene (CDNB) was determined as described by Habig and Jakoby [26] with a dual beam spectrophotometer (Perkin and Elmer, Lambda 3B). Hb concentrations in the samples were determined with the hemoglobin cyanide procedure described by van Kampen and Zijlstra [31].

Protein concentrations

Hepatocytes were lysed with ice cold water (1 : 6.7 v/v) and frozen until determination (-70°C). Protein concentrations were determined with the Bio-rad Protein Assay (BIO-RAD laboratories, München, Germany).

Statistical analysis

Significance of concentration dependent changes in $HbFe^{3+}$ accumulation and TBARS formation and changes in GT concentrations or in GST activity, were evaluated using linear regression analysis with a model including day of experiment and concentration of the test compound as explanatory variables. Using this model, we tested whether slopes of parameter changes versus concentration were significant correcting for the day of experiment.

Results

Hemoglobin oxidation

$HbFe^{3+}$ accumulation in *hemolysate* was linear for all three oximes over the concentration range tested ($p < 0.0001$ in all cases, Figure 6.1). CHO was the strongest oxidant (1.8 % $HbFe^{3+}$ accumulation for each mM CHO added) followed by AAO (1.2 %·mM^{-1} AAO) and MEKO (0.5 %·mM^{-1} MEKO). HYAM also caused a concentration dependent $HbFe^{3+}$ accumulation ($p < 0.0001$) and was much more potent than the oximes since hemoglobin was almost completely oxidized by 7 mM HYAM [16]. The oxo-compounds CH, AA and MEK caused no detectable $HbFe^{3+}$ accumulation.

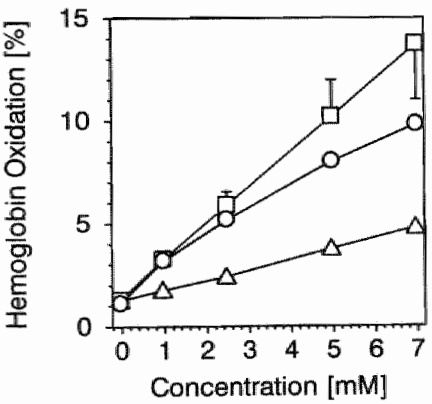

Figure 6.1: Hemoglobin oxidation in hemolysate (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

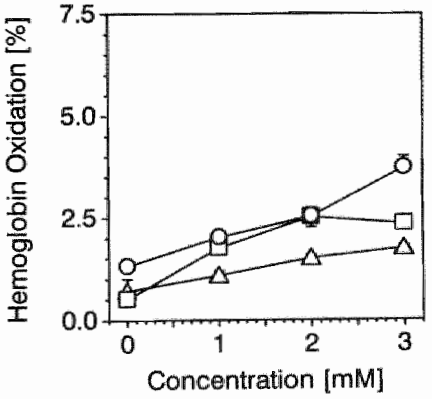

Figure 6.2: Hemoglobin oxidation in erythrocytes (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

In incubations with *erythrocytes* and oximes (Figure 6.2), $HbFe^{3+}$ accumulation was concentration dependent ($p < 0.01$ in all cases), but was lower than in hemolysates. When concentrations up to 2 mM were tested, CHO was the most

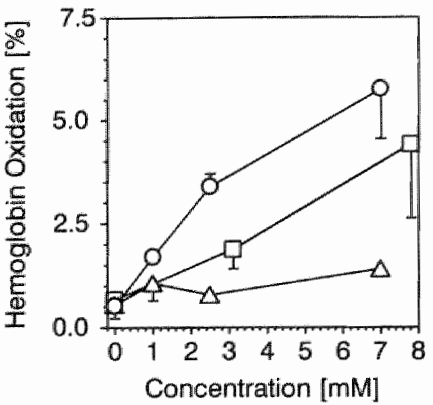

Figure 6.3: Hemoglobin oxidation in erythrocytes, after co-incubation of erythrocytes with rat liver microsomes (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

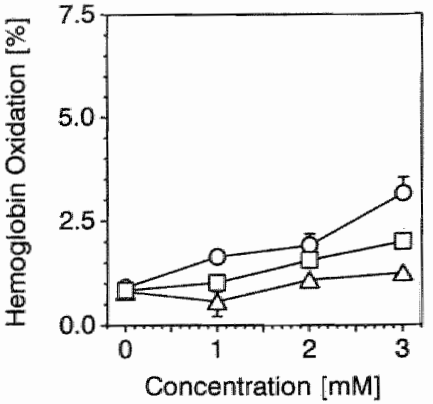

Figure 6.4: Hemoglobin oxidation in erythrocytes, after co-incubation of erythrocytes with rat hepatocytes (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

potent oxime (1.0 % $HbFe^{3+}$ accumulation for each mM CHO added), followed by AAO (0.6 %·mM^{-1} AAO) and MEKO (0.4 %·mM^{-1} MEKO). In contrast to AAO and MEKO, $HbFe^{3+}$ accumulation by CHO did not increase at concentrations higher than 2 mM. HYAM was again a stronger oxidant than the oximes. However, $HbFe^{3+}$ accumulation by HYAM in erythrocytes (88 % at 3 mM) was higher than in hemolysate (51 % at 2.5 mM). No $HbFe^{3+}$ was accumulated after the addition of CH and MEK to *blood*. $HbFe^{3+}$ accumulation caused by AA in *blood* was very low (0.05 %·mM^{-1} AA) but concentration dependent ($p = 0.0094$).

In co-incubations of *erythrocytes and microsomes* and oximes (Figure 6.3), AAO was the most potent hemoglobin oxidant (0.7 %·mM^{-1} AAO; $p = 0.0002$) followed by CHO (0.5 %·mM^{-1} CHO; $p = 0.0325$). The effect of MEKO on $HbFe^{3+}$ accumulation was very limited (0.1 %·mM^{-1} MEKO; $p = 0.090$).

When co-incubations of *erythrocytes and hepatocytes* and oximes were tested using transwells (Figure 6.4), $HbFe^{3+}$ accumulation was lower compared to the experiments with erythrocytes alone. AAO was the strongest oxidant (0.5 %·mM^{-1} AAO) followed by CHO (0.4 %·mM^{-1} CHO) when concentration up to 2 mM were tested ($p < 0.001$ in both cases). In contrast to incubations with erythrocytes alone, MEKO did not affect $HbFe^{3+}$ accumulation. HYAM was again a stronger oxidant than the oximes. $HbFe^{3+}$ accumulation by HYAM (81.0 % at 3 mM) was about the same as in the incubations with erythrocytes alone. Comparable results were obtained from co-incubations in which erythrocytes and hepatocytes were mixed.

When no oxime was added to an erythrocyte containing medium of an *isolated perfused rat liver*, no hemoglobin oxidation was found. Addition of CHO, AAO or MEKO (3 mM) to the perfusion medium caused an increase in $HbFe^{3+}$ accumulation of 2.9 %, 3.1 % and 0.9 % respectively, one hour after the start of the perfusion.

Lipid peroxidation

In incubations with *hemolysate* (Figure 6.5), TBARS formation was highest for AAO (221 nM (nano moles·l^{-1} TBARS for each mM AAO added), followed by AA, which is a possible metabolite of AAO (147 nM TBARS·mM^{-1} AA) ($p < 0.001$ in both cases). TBARS formation induced by HYAM was much lower than that caused by AAO and was maximal at 2.5 mM HYAM (241 nM; SE=25). CHO, MEKO, CH and MEK induced no TBARS formation in hemolysate.

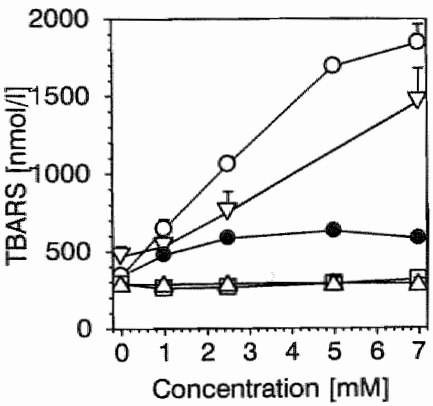

Figure 6.5: Formation of thiobarbituric acid reactive substances in hemolysate (incubation period 1 hour; 37°C). The effect of of □ CHO, ○ AAO, △ MEKO, ● HYAM and ▽ AA was tested.

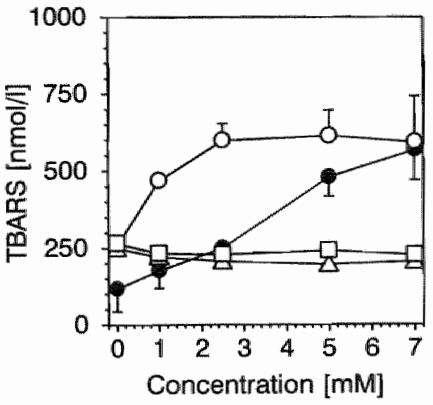

Figure 6.6: Formation of thiobarbituric acid reactive substances in human blood (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO, △ MEKO and ● HYAM was tested.

Incubation of *blood* with AAO or HYAM also caused TBARS formation (Figure 6.6). TBARS formation induced by AAO was maximal at 2.5 mM AAO (340 mM; SE=55). TBARS formation induced by HYAM was 78 nM TBARS·mM^{-1} HYAM and linear over the whole range of concentrations tested ($p < 0.0001$). The effect of AAO on TBARS formation was higher than the effect of HYAM, when test concentrations lower than 2.5 mM were tested. Contradictory to the incubations with hemolysate, AA caused no TBARS formation in blood. No detectable amounts of TBARS were released when blood was incubated with CHO, MEKO, CH and MEK.

TBARS concentrations were not determined in the co-incubations of erythrocytes and liver samples, as it is not possible to determine whether the effect is caused by erythrocytes or by the liver sample.

In incubations of *hepatocytes* with either oximes or HYAM, no TBARS release was detectable.

Glutathione

In incubations with *hemolysate* and AAO, MEKO and HYAM, GT was depleted in a concentration dependent manner ($p < 0.01$ in all cases, Figure 6.7). Remarkably, MEKO was the oxime which caused the highest GT depletion (0.20 μmoles per gram hemoglobin for each mM MEKO added up to a concentration of 5 mM). The effect of AAO on GT was very limited (0.06 μmoles·g^{-1}Hb·mM^{-1} AAO). GT was not affected by the addition of CHO. GT depletion was most pronounced when HYAM was tested (0.47 μmoles·g^{-1}Hb·mM^{-1} HYAM when concentrations up to 5 mM were tested). As with MEKO, GT depletion caused by HYAM did not increase further when concentrations higher than 5 mM were tested.

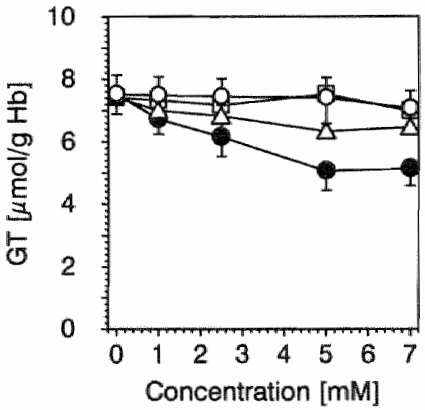

Figure 6.7: Total glutathione concentration in hemolysate (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO, △ MEKO and ● HYAM was tested.

No effect on GT was found when *erythrocytes* and oximes were tested. Addition of *rat liver microsomes* or *hepatocytes* to these erythrocyte incubations also gave no effect. A large fraction of the initial GT concentration in erythrocytes (6.0 μmoles·g^{-1} Hb; SE=0.3) was depleted when HYAM was tested (4.4 μmoles·g^{-1} Hb; SE=0.2 at 3 mM HYAM). Addition of hepatocytes to these incubations gave about equal results (4.7 μmoles·g^{-1} Hb; SE=0.2). GT concentrations in erythrocytes added to the medium of an *isolated perfused rat liver* were

not influenced by the addition of any of the three oximes tested (3 mM). GT was also not depleted when *hepatocytes* were incubated with the oximes and HYAM. CH, AA and MEK had no effect on GT in hemolysate, blood or hepatocytes.

Glutathione S-transferase

All three oximes inhibited GST activity in *hemolysate* in a concentration dependent manner ($p < 0.001$, Figure 6.8). GST inhibition caused by AAO was not linear and increased from 0.71 $U \cdot g^{-1}$ Hb (SE=0.2) at 1 mM AAO to 1.04 $U \cdot g^{-1}$ Hb (SE=0.2) at 7 mM AAO. CHO and MEKO inhibited GST activity in a concentration dependent way (0.27 $U \cdot g^{-1}$ Hb for each mM CHO added and 0.10 $U \cdot g^{-1} Hb \cdot mM^{-1}$ MEKO resp.). The inhibition of GST activity was most pronounced for HYAM. Like AAO, the greatest effect on GST activity was found at 1 mM HYAM (decrease of 2.38 $U \cdot g^{-1}$ Hb; SE=0.2). When 7 mM HYAM was tested, the inhibition of GST activity amounted to 3.12 $U \cdot g^{-1}$ Hb (SE=0.2). No effect on GST activity was found when CH, AA or MEK were tested.

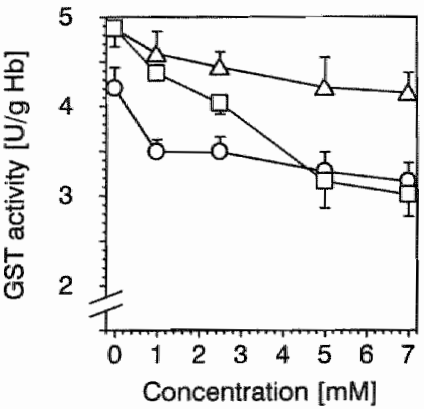

Figure 6.8: Glutathione S-transferase activity in hemolysate (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

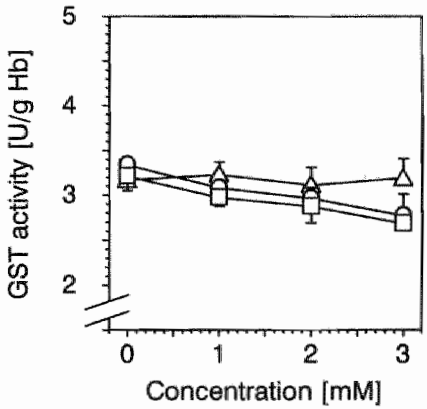

Figure 6.9: Glutathione S-transferase activity in erythrocytes (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

In incubations with *erythrocytes* and oximes (Figure 6.9), both CHO and AAO inhibited GST activity in a concentration dependent way ($p < 0.001$ in both cases). GST activity decreased with 0.17 $U \cdot g^{-1} Hb \cdot mM^{-1}$ CHO and with 0.18 $U \cdot g^{-1} Hb \cdot mM^{-1}$ AAO. MEKO had no influence on GST activity in erythrocytes. When HYAM was tested, GST inhibition amounted to 1.90 $U \cdot g^{-1}$ Hb (SE=0.9) at 3 mM HYAM. No effect on GST activity was found when CH, AA and MEK were tested in *blood*.

Inhibition of erythrocyte GST activity in co-incubations of *erythrocytes and microsomes* and oximes (Figure 6.10) was lower than in incubations with

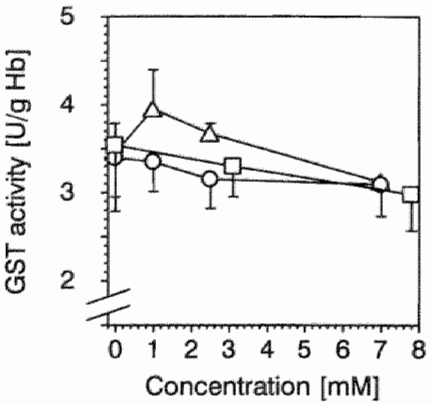

Figure 6.10: Glutathione S-transferase activity in erythrocytes incubated with rat liver microsomes (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

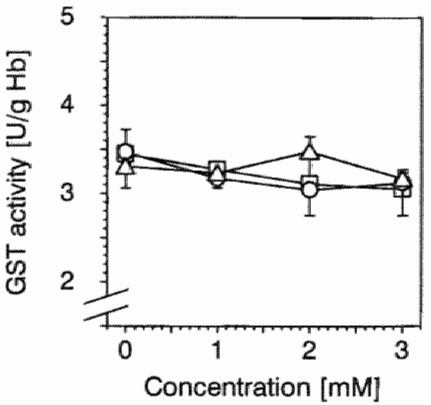

Figure 6.11: Glutathione S-transferase activity in erythrocytes incubated with hepatocytes (incubation period 1 hour; 37°C). The effect of □ CHO, ○ AAO and △ MEKO was tested.

erythrocytes alone. The GST activity decreased with 0.04 $U \cdot g^{-1}Hb \cdot mM^{-1}AAO$ ($p = 0.0296$). The effect of CHO and MEKO on GST activity was not statistically significant.

Inhibition of erythrocyte GST activity caused by oximes in co-incubations of *erythrocytes and hepatocytes* (Figure 6.11) was lower than in incubations with erythrocytes alone. GST inhibition caused by CHO was 0.13 $U \cdot g^{-1}Hb \cdot mM^{-1}$ CHO ($p = 0.0819$). AAO and MEKO had no influence on erythrocyte GST activity.

No time-dependent effect on GST activity in human erythrocytes could be observed when oximes were added to the erythrocyte containing medium of an *isolated perfused rat liver*. None of the substances tested (CHO, AAO, MEKO, HYAM, CH, AA and MEK) had a detectable effect on GST activity in *hepatocytes* at a concentration of 7 mM.

Discussion

Exposure of rats to the oximes CHO and MEKO causes hemolytic anemia [13, 21, 34]. It is not well understood whether the hematotoxic effects of these oximes are due to the parent compounds per se or to their metabolites. Parmar and Burka [42] showed that CHO can be metabolised into CH and then reduced to cyclohexanol. They suggested that CH is the active metabolite of CHO as CH induces hematotoxic effects. However, HYAM is also released during hydrolysis

of CHO and may likewise be responsible for the hematotoxic effects of CHO. In humans many arylhydroxylamines are hemolytic agents that cause methemoglobinemia [17, 30]. Hydroxylamine and other related compounds not only lead to methemoglobin formation but also produce GT depletion in erythrocytes *in vitro* [7, 23]. In addition to the hydrolytic breakdown of oximes, two *anaerobic* metabolic pathways have been reported; 1) liver aldehyde oxidase metabolizes oximes into ketimines in the presence of the electron donor 2-hydroxypyrimidine. These ketimines will be hydrolyzed nonenzymatically into an oxo-compound and ammonia [50]; no HYAM will be formed in this way 2) acetophenone oxime is reduced by both microsomal and cytosolic enzymes yielding HYAM as one of the products [27]. According to these authors, acetophenone oxime was stable to oxidative and hydrolytic transformations. In contrast, Kohl *et al.* [32] found that acetoxime is *oxidized* by human, rat and mice hepatic cytochrome P-450 1A, 2B and $2E_1$ into propane 2-nitronate, which is a potent genotoxicant and carcinogen, and than reduced into 2-nitropropane. Both acetoxime and 2-nitropropane cause elevated levels of 7-hydroxyguanine in liver DNA and RNA in rat [10, 11, 19, 20, 25].

In this study we tested on one hand whether the oximes CHO, AAO and MEKO as such are able to generate oxidative effects in human erythrocytes, and on the other hand whether the suggested metabolites of these oximes, CH, AA, MEK, respectively and HYAM are hematotoxic. Oximes were also tested in a combined assay of human erythrocytes with microsomes or hepatocytes, and in an isolated rat liver perfusion in which human erythrocytes were added to the medium to investigate whether possible metabolites generated in these test systems are able to generate oxidative effects in erythrocytes and/or hepatocytes.

$HbFe^{3+}$ accumulation, TBARS formation, GT depletion and GST activity changes were tested. We chose these parameters since a) *in vivo* exposure to CHO causes hemoglobin oxidation and Heinz body formation [13, 21] b) we previously found that HYAM, a possible metabolite of oximes, affects all four parameters [16]. During the process of $HbFe^{3+}$ formation, free radicals are formed [49]. These free radicals may be the initiators for the TBARS formation that occurred. They can also give rise to the generation of protein disulfides, mixed disulfides or oxidized glutathione [24]. Lipid peroxides can be reduced into alcohols by glutathione peroxidase or glutathione S-transferase [18], and protein disulfides or mixed disulfides can be reduced by thioltransferase, with concomitant oxidation of GSH [36]. These processes may lead to a reduction of erythrocyte GSH levels [18, 33]. Erythrocyte GST, which is a π-class enzyme, is known to be vulnerable to oxidative stress [14, 16, 45, 46] and electrophiles [6, 35], and was therefore also included as an effect parameter in the experiments.

The results show that the oximes and HYAM all caused hemoglobin oxidation in erythrocytes. Although the $HbFe^{3+}$ accumulation for HYAM was not higher in

hemolysate than in erythrocytes, this was clearly the case for the oximes. This indicates that uptake of the oximes by the erythrocytes may be a limiting factor. The magnitude of hemoglobin oxidation by oximes in erythrocytes was low compared to HYAM and lowest for MEKO. This was also the case in hemolysates. Therefore, although uptake limitation cannot be ruled out, it is not the only factor determining potency differences between oximes and HYAM. Incubation of the possible oxime metabolites CH, AA or MEK caused no $HbFe^{3+}$ accumulation, showing that, even if CH, AA and MEK are metabolites of the oximes, they are not responsible for methemoglobin formation. Co-incubation of human erythrocytes and rat liver preparations (i.e. microsomes, hepatocytes, whole liver) did not affect $HbFe^{3+}$ accumulation by oximes. However, possible metabolites generated by hepatocytes may not be able to reach their target in the erythrocyte. No effects on hepatocyte GT depletion, TBARS formation and GST activity were found in incubations of hepatocytes with CHO, AAO or MEKO. All in all, this indicates that the formation of reactive oxime metabolites by hepatocytes is of minor importance for oxime activation. A comparison between the *in vitro* and *in vivo* results [13] concerning $HbFe^{3+}$ accumulation is difficult. Although the *in vivo* intravascular CHO concentration is not known, it was probably in the millimolar range. Also, the longer duration of the *in vivo* experiments may have lead to cumulative effects. Sequestration of erythrocytes containing high amounts of $HbFe^{3+}$ accumulated in the spleen may have obscured these effects. Furthermore, a difference in vulnerability may exist between human and rat erythrocytes.

Lipid peroxidation was measured as TBARS production. TBARS production in human blood was found only for AAO and HYAM. Opposed to what was found for HYAM, the TBARS formation caused by AAO was much higher in hemolysate than in blood. Uptake limitation for AAO in the erythrocyte may be the reason for this. AA, a possible metabolite of AAO only caused a detectable TBARS formation in hemolysate. AA is a substrate for xanthine oxidase, and the generation of free radicals in this reaction [39], may explain the TBARS formation in hemolysate. However, additional experiments with the xanthine oxidase inhibitor allopurinol showed that the role of xanthine oxidase activity in the induction of TBARS formation by AA in hemolysate is negligible (data not shown). The magnitude of the TBARS formation caused by AAO found in hemolysate was about the sum of the effects found for AA and HYAM. Therefore, hydrolysis of AAO to AA and HYAM could explain the TBARS formation inducing activity of AAO. In hepatocytes, no TBARS were formed by oximes or HYAM. This together with the known generation of free radicals during the $HbFe^{3+}$ formation by HYAM [49] leads to the conclusion that HYAM needs hemoglobin to induce TBARS formation. This was confirmed by the observation that no TBARS were formed when HYAM was incubated with washed red blood cell membranes (data not shown). Considering AAO, a factor from the erythrocytes also seems to be required for TBARS formation as AAO caused no TBARS in hepatocytes.

Glutathione (GT) in erythrocytes was depleted only by HYAM. In hemolysate, AAO and MEKO as well as HYAM caused GT depletion. However, the magnitude of the effect caused by AAO was small. Comparing the magnitude of the effects caused by HYAM on erythrocyte $HbFe^{3+}$accumulation, TBARS and GST, erythrocyte GT depletion was the least sensitive parameter. As the effects of oximes on $HbFe^{3+}$ accumulation, TBARS and GST were much lower than the effects of HYAM, we expected no effect of the oximes on erythrocyte GT. Interestingly, MEKO, which was the only oxime that did not cause GST inhibition in erythrocytes, did give rise to GT depletion. The most probable explanation for this GT depletion is glutathione conjugation of MEKO or MEKO metabolites that may be formed by the cytosol of erythrocytes. No GT depletion was found when hepatocytes or microsomes were added to the incubations with erythrocytes, which is in accordance with the fact that $HbFe^{3+}$ accumulation was generally not influenced by the addition of liver preparations. Hepatic glutathione was also not affected by oximes or HYAM. As hepatocytes contain about 10 mM glutathione [9] and erythrocytes only about 2 mM, the higher glutathione concentration in hepatocytes may mask GT depletion. The fact that GT depletion caused by oximes was not higher in erythrocytes after the addition of liver preparations confirms that no important formation of erythrotoxic reactive metabolites occurs under these experimental conditions.

Glutathione S-transferase (GST) activity decreased when erythrocytes were incubated with CHO, AAO or HYAM. The effect was more pronounced when hemolysate was tested, and in this case even MEKO induced a decrease in GST activity. GST-π (comparable to erythrocyte GST-ρ) is known to be vulnerable for free radicals and lipid peroxidation products [14, 16, 45, 46]. The strong erythrocyte GST inhibition caused by HYAM or AAO (both compounds induced TBARS formation) can be a consequence of free radicals or toxic products generated during TBARS formation. The small GST inhibitory capacity of MEKO in incubations with hemolysate may be a consequence of marginal TBARS formation. CHO and AAO inhibited erythrocyte GST to the same extent. However, CHO caused no TBARS formation in erythrocytes. For this reason we expected to find a GST inhibition which was comparable to the GST inhibition found for MEKO. As the erythrocyte GST inhibition caused by CHO was much higher, inhibition of GST works via a different mechanism from that found for AAO and HYAM. Many compounds are known to inhibit GST activity directly by modification of the enzyme [35]. CHO may function in the same way. All effects on GST were lower when microsomes or hepatocytes were added to the incubations with erythrocytes. This could be a result of impaired availability of the test compound caused by absorption by the hepatocytes or microsomes (e.g. protein binding).

Based on our *in vitro* results, we conclude that a) oximes have less oxidative capacity than HYAM. b) AAO may be regarded as more erythrotoxic than CHO and MEKO, as it causes TBARS formation. c) hemoglobin oxidation, TBARS

formation and GST activity decrease are generally related to each other. However, specific GST inhibition (e.g. CHO) disturbs this general relationship. d) compared to $HbFe^{3+}$ accumulation and GST inhibition, GT depletion is a less sensitive parameter for oxidative compounds and may be strongly influenced by compounds metabolised by GSH conjugation, as is possibly the case for MEKO e) the oxo-compounds related to the oximes tested gave no effect with the exception of AA in TBARS formation f) the inclusion of liver preparations had no effect on erythrocyte parameters. This indicates that liver metabolism is not of major importance for the hematotoxic effects of oximes.

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Chapter 7

General discussion

Blood is the fluid circulating through the body carrying nutrients and oxygen through erythrocytes and plasma to body cells and removing waste products and carbon dioxide. During its circulation through the arteries and veins, erythrocytes come in close contact with organs. One of these organs is the liver. The liver has the highest metabolic capacity and as metabolites generated by the hepatocytes may leave these cells, erythrocytes can be exposed to products of biotransformation. As a result of this exposure, targets or biochemical reactions in erythrocytes may be affected.

In this thesis, the interplay between erythrocytes and hepatocytes was studied with special attention to bio-activation and inactivation capacity of both liver and blood. In order to gain an insight in this problem, several *in vitro* techniques had to be considered and developed.

The primary reasons to use human blood cells were (i) to facilitate extrapolation from the *in vitro* to the human *in vivo* situation and (ii) that blood may be applicable in biological effect monitoring of workers exposed to chemicals. Moreover, the erythrocyte is a relatively vulnerable blood cell since it possesses no nucleus and organelles [11], so it is totally dependent on glycolysis for its need of ATP [28]. The absence of a nucleus and endoplasmic reticulum makes that erythrocytes cannot synthetize proteins. This means that loss of enzyme activity is irreversible since it cannot be compensated by the synthesis of new enzyme. The transport of oxygen, which is the most important function of erythrocytes, is dependent on ferrous hemoglobin. Oxidized hemoglobin can be reduced by means of NADH cytochrome b_5 reductase and NADPH flavin reductase using NADH and NADPH respectively, as reducing equivalents [21, 31, 32]. Moreover, the reductant NADPH is needed in several other biochemical reactions (e.g. reduction of oxidized glutathione [15, 17]). The need for NADPH in the erythrocyte is totally fulfilled by the hexose monophosphate shunt.

The effect parameter which was studied most intensely in this thesis the is glutathione content. Glutathione is a tripeptide present both in erythrocytes and hepatocytes. Cellular glutathione stores can be depleted by means of conjugation

of reduced glutathione (GSH) to electrophilic substrates, the generation of mixed disulfides [10] or by export of oxidized glutathione (GSSG) from the cell [1, 13]. Several target systems containing GSH as a molecular target were used (Figure 7.1). The results obtained in this thesis using different GSH sources and substrates are summarized in Table 7.1.

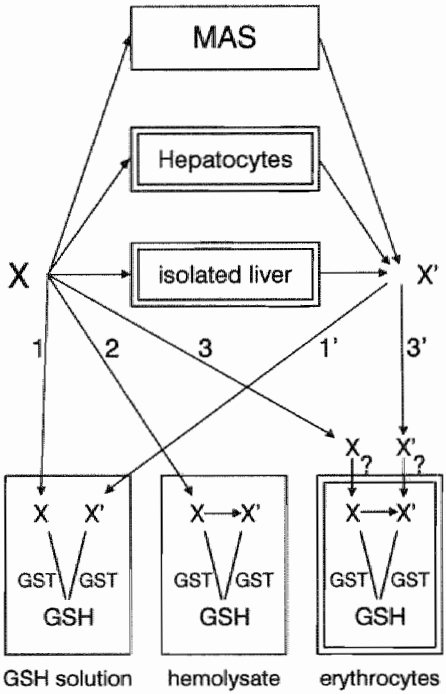

Figure 7.1: An outline of the incubations performed in this thesis. The picture is explained in the text. 1) glutathione solution 2) hemolysate 3) erythrocytes 1') glutathione solution plus microsomal activating system (MAS) or hepatocytes 3') erythrocytes plus MAS, hepatocytes or whole liver

The ability of several compounds with different mechanisms of action to react with GSH was tested in incubations with GSH solution (arrow 1). We found that iodoacetamide, N-ethylmaleimide and diethyl maleate strongly depleted the amount of GSH in solution (see also Table 7.1). These compounds were also able to deplete erythrocyte GSH (arrow 3) which means that they can pass the erythrocyte membrane. Another group of compounds is not able to deplete total glutathione (GT, which is the sum of both GSH and GSSG) in incubations with GSH solution. Cyclophosphamide, 3-hydroxyacetanilide, 2-methylfurane, monuron and monulinuron belong to this group. Erythrocytes are metabolically

Table 7.1: Qualitative summary of the test results on glutathione depletion, obtained with the different test systems and substrates used in this thesis.

Substrate + (Chapter)	GSH solution −MAS	GSH solution +MAS	Hemo-lysate	Erythrocytes −MAS	Erythrocytes +MAS	Erys + Hep	Perfu-sion
IAcA (3)	+			+			
NEM (3)	+			+			
DEM (3)	+			+			
HYAM (6)			+	+		+	
CP (2,3)	0	+	0	0	+		
3-HAA (2,3)	0	+	+	0	+	0	
2-MF (3)	0	+	+	0	+		
MN (3)	0	0	0	0	0		
MLN (3)	0	0	0	0	0		
CHO (6)			0	0	0	0	0
AAO (6)			+/0	0	0	0	0
MEKO (6)			+	0	0	0	0

Glutathione depletion was measured in incubations with reduced glutathione (GSH), hemolysate or erythrocytes. The effect of addition of (i) microsomal activating system of normal rats (MAS) to incubations with GSH solution or erythrocytes (ii) hepatocytes to erythrocytes (Erys+Hep) or (iii) a whole liver perfused with erythrocytes (perfusion) was also tested. Depletion of glutathione by a compound is indicated with (+), a marginal effect is indicated as (+/0) and no effect on glutathione as (0). Testsubstrates were (IAcA) iodoacetamide, (NEM) N-ethylmaleimide, (DEM) diethyl maleate, (CP) cyclophosphamide, (HYAM) hydroxylamine, (3-HAA) 3-hydroxyacetanilide, (2-MF) 2-methylfurane, (MN) monuron, (MLN) monulinuron, (CHO) cyclohexanone oxime, (AAO) acetaldoxime and (MEKO) methyl-ethyl ketoxime.
Significance of glutathione depletion was determined by linear regression of the slope of GT depletion versus concentration of the substrate. The substrate concentrations tested differed between compounds and are described in the respective chapters. The samples were incubated for 1 hour at 37°C. Depletion of reduced glutathione was measured when IAcA, NEM and DEM were tested. Total glutathione depletion was measured for the other compounds.

active [3, 5, 16, 19, 20, 29]. If compounds belonging to the group described above are also metabolically activated in the erythrocytes, they may afterwards be able to deplete GT in these incubations. However, no GT depletion in erythrocytes was found when cyclophosphamide, 3-hydroxyacetanilide, 2-methylfurane, monuron and monulinuron were tested. As the amount of compound that passed

the erythrocyte membrane during the 1 hour incubation period may be too low because of diffusion limitation, hemolysates were also incubated with the non-reactive compounds. Both 3-hydroxyacetanilide and 2-methylfurane depleted the GT content in hemolysates. This may indicate that GSH-reactive metabolites are formed by the hemolysates. However, the absence of a cellular membrane may not be the only difference between erythrocytes and hemolysate. Cyclophosphamide did not affect the GT concentration in hemolysates. The strong oxidative compound hydroxylamine was able to deplete the GT concentration in both incubations with erythrocytes and hemolysate. Since oximes might be metabolised by erythrocytes into hydroxylamine and the respective oxo-compound, GT reactive metabolites might be formed. However, no GT depletion was detected in incubations with erythrocytes and cyclohexanone oxime, acetaldoxime and methyl-ethyl ketoxime. However, methyl-ethyl ketoxime and, to a marginal extent, acetaldoxime were able to deplete GT in incubations with hemolysate. The difference in results obtained with erythrocytes and hemolysates may not only be due to the membrane but also to for instance differences in reductive capacity due to higher oxygen exposure of the hemolysates.

To study the interplay between hepatic and erythrocyte metabolism several *in vitro* techniques were used in which rat liver derived tissue was added to incubations with GSH solution or erythrocytes (Figure 7.1, arrow 1' and 3'). Rat liver was used because it is very difficult to obtain human livers. A disadvantage of the use of rat tissue is that the biotransformation pathways between rat and man may be different, so that the results obtained in the incubations using rat liver derived tissues may not apply to the human situation. The test compounds which did not deplete GT in the incubations with GSH solution and erythrocytes were tested again in these systems but now in the presence of rat liver microsomal activating system (MAS). Cyclophosphamide, 3-hydroxyacetanilide and 2-methylfurane were metabolised by MAS into GSH-reactive compounds as GT was depleted both in incubations with GSH solution and erythrocytes (Table 7.1). Implicitly this means that the metabolites generated by MAS were able to pass the erythrocyte membrane. The generation of the GSH-reactive metabolites was NADPH dependent which is indicative of activity of the cytochrome P-450 complex. GT depletions were increased when microsomes derived from rats pretreated with phenobarbitone were added. This indicates that the phenobarbitone inducable enzymes, which include some isoenzymes of cytochrome P-450, also participate in the generation of the GSH-reactive cyclophosphamide, 3-hydroxyacetanilide and 2-methylfurane metabolites. GT was not depleted when the pesticides monuron and monulinuron were tested in incubations with GSH solution or erythrocytes with or without the addition of MAS. The three oximes were also not metabolised by MAS into detectable amounts of GSH-reactive metabolites. Although there is no definitive explanation for this phenomenon, the following options may be the case (i) no oxime metabolites are formed by the liver (ii) possible metabolites

formed in the test system are not able to pass the erythrocyte membrane or (iii) do not conjugate with GSH.

A test system more closely imitating the physiological situation than erythrocytes and MAS, consisted of co-incubated human erythrocytes and rat hepatocytes (Figure 7.1, arrow 3'). In contrast to the previous systems, GSH-reactive metabolites in this model must first cross the hepatocyte and Transwell^{TM} membrane to reach erythrocyte targets. Rat hepatocytes are a metabolically very active cell type but they also have a high detoxification capacity [8]. This means that GSH-reactive metabolites may be formed by hepatocytes but also that they may be detoxified within the hepatocyte. As a result of this intracellular detoxification, less GSH-reactive metabolites will leave the hepatocyte. Cyclophosphamide and 3-hydroxyacetanilide were metabolised by hepatocytes into GSH-reactive compounds since they depleted the amount of intracellular GT (Chapter 3). Interestingly, the GSH-reactive metabolites of both cyclophosphamide and, notably, 3-hydroxyacetanilide generated by the hepatocytes were also excreted into extracellular milieu (Chapter 3). For this reason, 3-hydroxyacetanilide was tested in co-incubations of hepatocytes and erythrocytes. Metabolites generated by the hepatocytes were expected to pass the hepatocyte and the erythrocyte membrane to deplete erythrocyte GT. However, no effect on erythrocyte GT could be detected, which may be explained by uptake limitation of the GSH-reactive metabolite(s) into the erythrocyte (Table 7.1). Also no GT depletion was found when cyclohexanone oxime, acetaldoxime and methyl-ethyl ketoxime were tested in co-incubations of hepatocytes and erythrocytes. Oximes may be metabolised by hepatocytes into hydroxylamine, a strongly oxidative compound, able to pass the hepatocyte and the erythrocyte membrane and oxidize erythrocyte GSH in previous experiments. Since neither erythrocyte nor hepatocyte GT were affected by the oximes this indicates that no GSH-reactive oxime metabolites are formed in the hepatocytes.

The *in vitro* technique closest to the *in vivo* situation was the isolated perfused liver. Since no effects on erythrocyte GT were found in co-incubations of erythrocytes and hepatocytes testing 3-hydroxyacetanilide or the oximes, it was not expected that erythrocyte GT would be affected in the liver perfusions. This was confirmed in liver perfusion experiments in which the oximes were tested (Table 7.1).

Oximes are known oxidative compounds *in vivo* that may be metabolised in the body into oxo-compounds and hydroxylamine. In addition to GT depletion, hydroxylamine can oxidize hemoglobin, cause lipid peroxidation and affect erythrocyte GST activity [7]. Therefore, these parameters were also assessed in our studies on the effects of oximes on human erythrocytes. In fact, a detailed comparison of several effect markers showed that depletion of GT is a relatively insensitive parameter to evaluate oxidative effects in blood (Table 7.2). $HbFe^{3+}$

Table 7.2: Hemoglobin oxidation, lipid peroxidation, glutathione depletion and inhibition of glutathione S-transferase activity in hemolysate and erythrocytes caused by oximes and hydroxylamine.

	hemolysate				erythrocytes or blood			
Substrate	$HbFe^{3+}$	TBARS	GT	GST	$HbFe^{3+}$	TBARS	GT	GST
HYAM	+	+	+	+	+	+	+	+
CHO	+	0	0	+	+	0	0	+
AAO	+	+	+/0	+	+	+	0	+
MEKO	+	0	+	+	+	0	0	0

($HbFe^{3+}$) hemoglobin oxidation, (TBARS) formation of thiobarbituric reactive substances, (GT) total glutathione depletion and (GST) glutathione S-transferase activity changes caused by different substrates were tested in incubations with hemolysate and erythrocytes or blood. When the parameter tested is affected by the test compound this is indicated by (+). No effect of the test compound or a marginal effect are indicated by (0) and (+/0) respectively. Testsubstrates were (HYAM) hydroxylamine, (CHO) cyclohexanone oxime, (AAO) acetaldoxime and (MEKO) methyl-ethyl ketoxime. In contrast to the other parameters in which washed erythrocytes were used, formation of TBARS was tested in whole blood. Samples were incubated for 1 hour at 37°C. Statistical significance of the effect found was determined by linear regression of the slope of the substrate concentration versus the parameter measured. Substrate concentrations up to 7 mM were tested in incubations with hemolysate. In incubations with erythrocytes or blood, substrate concentrations up to 3 mM were tested except for TBARS formation in blood which was tested up to 7 mM.

formation and GST inhibition were much better parameters as they were generally affected by the oximes while GT was only markedly depleted by methyl-ethyl ketoxime in hemolysate and hydroxylamine in both erythrocytes and hemolysate. Although $HbFe^{3+}$ formation was most pronounced when oximes were incubated with hemolysate, also in erythrocytes $HbFe^{3+}$ formation was observed. During the generation of oxidized hemoglobin, free radicals are formed [27] that may initiate lipid peroxidation. The formation of lipid peroxides was determined by means of thiobarbituric acid reactive substances (TBARS) [26]. The only oxime that caused detectable TBARS in erythrocytes and hemolysate was acetaldoxime. Therefore, lipid peroxidation is considered as a less sensitive general effect parameter, because only one of the three oximes tested caused lipid peroxidation. However, it does give more specific information about the ability of a compound to initiate lipid peroxidation. Erythrocyte GST activity was lowered after incubation of hemolysate with all three oximes or after incubation of cyclohexanone

oxime or acetaldoxime with erythrocytes. This can be explained by the fact that free radicals or products of lipid peroxidation can inhibit erythrocyte GST activity [6,7,23,24]. The effects found in the incubations with erythrocytes alone were not enlarged when metabolizing systems were added. This indicates that liver metabolism is not of major importance for the hematotoxic effects of oximes.

In conclusion, glutathione depletion is a good parameter when strong electrophilic compounds are tested or when electrophilic metabolites are generated by metabolizing systems. GT depletion can also be used as an effect parameter when strong oxidative compounds are tested, but for this category of compounds there are more sensitive options (e.g. hemoglobin oxidation or GST inhibition). GT depletion is not a sensitive parameter when weak oxidative compounds are tested. In this case, hemoglobin oxidation and GST inhibition showed to be preferable (Table 7.2). As free radicals are generated during the process of $HbFe^{3+}$ formation caused by hydroxylamine, and oximes may be hydrolyzed into hydroxylamine, the effects of the oximes on GST activity and TBARS formation may be a result of this $HbFe^{3+}$ formation.

Apart from being a target to reactive metabolites generated and released from tissues, erythrocytes are specific oxygen carriers. Our studies testing the isolated perfused rat liver without oxygen carriers showed degeneration of liver tissue (Chapter 5), especially affecting the perivenous hepatocytes. As the cytochrome P-450 content is high in the perivenous hepatocytes [9,22,30], the usage of medium without oxygen carriers may therefore affect the capacity to oxidize xenobiotics. Also oxygen deprivation that does not lead to degeneration of hepatocytes may lead to a reduced metabolic activity of cytochrome P-450 [2]. However, the isolated liver was still able to metabolize dimethylacetamide (DMAc) which was added to a medium composed of bicarbonate buffered Krebs solution plus 1 % albumin and the elimination velocity constant of DMAc was determined (Chapter 4). DMAc was expected to be metabolised into N-hydroxymethyl N-methylacetamide that may be oxidized further into monomethylacetamide [4,12,18,25]. However, monomethylacetamide could not be detected in the perfusates. Since addition of erythrocytes to the medium might influence the capability of the liver to metabolize DMAc, the elimination velocity constant of DMAc was determined in perfusions in which human erythrocytes were added to the perfusion medium (Chapter 5). However, no significant difference was found between the elimination velocity constant of DMAc determined in perfusions with or without human erythrocytes added to the perfusion medium. It was not determined whether human erythrocytes were able to metabolize DMAc. However, if DMAc is metabolised by erythrocytes than the elimination velocity constant in perfusions with erythrocytes would have been higher. It can be concluded that the liver in an isolated perfused liver system metabolizes DMAc and that human erythrocytes have a positive influence on the viability of the liver cells.

The techniques described in this thesis can be used to evaluate the ability of a compound and/or its metabolites to affect erythrocytes. As it is important to measure the proper targets in erythrocytes, it is recommended to study the chemical properties of a compound and/or its metabolites prior to the choice of the parameters that will be measured. As already mentioned, GT depletion may be a good target when electrophiles have to be detected. In case of weakly oxidative compounds, hemoglobin oxidation and GST inhibition are better parameters to be measured. If an insight into the ability of a compound to affect erythrocyte parameters is wanted, this can be tested in incubations with erythrocytes. Beside a direct effect of the parent compound on the parameter tested also metabolites generated by the erythrocytes may be responsible for the effects found. If GT depletion is chosen as an effect parameter, incubation of the test compound with GSH solution is an option to circumvent the possibility of metabolite generation by erythrocytes. The ability of metabolites generated by rat liver preparations to affect erythrocyte parameters can be tested in co-incubations of erythrocytes and rat liver derived metabolizing systems. Since metabolites generated by microsomes do not have to pass a hepatocyte membrane and as there is no conjugation with endogenous substrates, a greater effect on erythrocyte parameters is expected to be found in co-incubations of erythrocytes and MAS compared to co-incubations in which hepatocytes or a whole liver are tested. This was was confirmed in the studies performed in this thesis.

Co-incubations of erythrocytes and hepatocytes may give an insight into the ability of metabolites generated by hepatocytes to leave the hepatocyte, enter the erythrocyte and affect the erythrocyte parameters tested. The metabolites are probably transported over the membranes by diffusion. One of the factors determining the amount of metabolite that passes the erythrocyte membrane by diffusion is the amount of metabolites generated and excreted by the hepatocytes. In this way, the concentration gradient over the erythrocyte membrane determines the intra-erythrocyte metabolite concentration that may give a detectable change in the erythrocyte parameter(s) measured. Beside the concentration gradient over the membrane, also the time of incubation affects the amount of metabolite(s) that passes the erythrocyte membrane. In Chapter 3, the diffusion equations described by Fick were used to gain insight into the permeability coefficient of the metabolite(s) of 3-hydroxyacetanilide. Glutathione conjugation was the effect parameter tested. Since the reaction between glutathione and the metabolite formed is equimolar, the minimal amount of metabolite that had to be formed per unit of membrane and time so that glutathione depletion could be detected was calculated. Using this value, the permeability constant that must minimally be reached to find glutathione depletion was calculated for the metabolite concentration which was found outside the erythrocytes.

The incubations performed in this thesis can be extended with ghosts to test whether hemoglobin plays an essential role in the generation of TBARS when ery-

throcytes or hemolysate are incubated with acetaldoxime. Furthermore, ghosts may also be filled with target systems that may be affected by a xenobiotic or its metabolite(s). The incubations with Transwells^{TM} can be improved by increasing the concentration gradient over the erythrocyte membrane. This can be done by increasing the amount of hepatocytes added to the system so that the amount of metabolites generated and excreted by the hepatocytes may increase. The amount of metabolites formed by the hepatocytes may also be increased by pretreatment of rats with enzyme inducers. Since the metabolites generated by the hepatocytes were diluted to a great extent as a consequence of the technical conditions set by the Transwells^{TM}, concentration of the test system by using smaller volumes in which the erythrocytes and hepatocytes are diluted may also help. Another possibility to enlarge the sensitivity of this test system is increasing the time of incubation. In this way, higher amounts of metabolites may be formed and the amount of metabolite that passes the erythrocyte membrane by diffusion increases. However, it may be necessary to use sterile techniques when the incubation period has to be extended. However, incubation periods of more that 24 hours are not expected to be effective as cytochrome P-450 activity of hepatocytes decreases drastically with time [14]. The effects found in human erythrocytes that are perfused through an isolated rat liver may be increased by means of the same variables mentioned for Transwells^{TM}.

Based on the results obtained in this thesis it can be concluded that erythrocyte parameters were affected by several test compounds. Which parameter(s) was/were affected depended on the compound tested. In *in vitro* incubations with hemolysate the effects were higher than in incubations with erythrocytes. Hemolysate was able to generate a detectable amount of GSH-reactive metabolites out of some test compounds. This was not the case when the same substrates were tested in incubations with erythrocytes. GSH or GT depletion was a good effect parameter when electrophilic or strong oxidative compounds were tested, but not when weak oxidative compounds had to be detected. In that case hemoglobin oxidation or changes in GST activity were better options. Since compounds not able to affect erythrocyte parameters might be able to do so after metabolization by rat liver preparations, co-incubations of erythrocytes and MAS or hepatocytes, and perfusion of erythrocytes through an isolated rat liver were performed. Test substrates which were able to affect erythrocyte parameter(s) after metabolic activation were only able to do so after co-incubation with MAS. No extra effect on erythrocyte parameters caused by hepatocytes or a total liver could be detected after co-incubation of erythrocytes with hepatocytes or after perfusion of erythrocytes through an isolated liver. This may be explained by detoxification of reactive metabolites in the hepatocytes or by limited diffusion of reactive metabolites over the hepatocyte membrane. Although perfusion through an isolated liver of medium enriched with erythrocytes did not increase the effects found in incubations with erythrocytes alone, erythrocytes on their

turn played an important role in the maintenance of liver cell viability. Since no effects were found in the test systems that approximated the *in vivo* situation more than MAS, this indicates that the sensitivity of the test systems tested in this thesis decreased with the complexity of the test system. This may be explained by the capacity of hepatocytes and the total liver to adapt to changing circumstances.

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Chapter 8

Summary

Blood plays an important role in the body because of its transport function. The transport of oxygen to the tissues and of carbondioxyde to the lungs is confined to erythrocytes. Because of their central role, erythrocytes come in close contact with organs that may absorb foreign compounds (xenobiotics) and with organs that may transform non-toxic compounds into reactive metabolites. Erythrocytes also have metabolic activity themselves. The cells contain several mechanisms of protection, usually of biochemical origin, against toxic compounds or their metabolites. Cell damage may occur when the balance between the stress erythrocytes are exposed to and the capacity of detoxification is disturbed.

In the liver erythrocytes come in close contact with the metabolically very active hepatocytes. Non-toxic compounds may be metabolised by hepatocytes into toxic metabolites that may affect the erythrocytes. In this thesis main attention is directed to the interplay between erythrocytes and hepatocytes with special emphasis to the bioactivation and inactivation capacity of liver and blood.

Glutathione plays a role in the protection of erythrocytes against electrophilic and oxidative stress. *Chapters 2 and 3* show that strong electrophilic compounds that are able to pass the erythrocyte membrane (iodoacetamide, N-ethylmaleimide and diethyl maleate) were able to deplete the concentration of reduced glutathione (GSH) in erythrocytes. Since the test compounds also depleted GSH in incubations with GSH dissolved in buffer (GSH solution), this means that they are able to deplete GSH without prior metabolic activation. From the experiments with hemolysate we conclude that 3-hydroxyacetanilide (3-HAA) and 2-methylfurane (2-MF) are metabolized by hemolysate into GSH-reactive compounds. The total glutathione concentration ((GT) which is the sum of both GSH and oxidized glutathione) in hemolysate decreased with the concentration of the test compound while the GT concentration was not affected in incubations with GSH solution. Both 3-HAA and 2-MF did not affect erythrocyte GT which may be explained by a low intracellular substrate concentration. Since hemolysate is not the same as erythrocytes without a cellular membrane, the difference in effect between incubations with erythrocytes and hemolysate cannot be explained only by these

cellular membranes. Compounds that cannot deplete erythrocyte GT may be able to do so after extracellular metabolic activation. Both cyclophosphamide (CP), 3-HAA and 2-MF were able to deplete erythrocyte GT after metabolic activation by means of rat liver microsomal activating system (MAS) indicating that the metabolites were able to cross the erythrocyte membrane. The GT depletion in erythrocytes was higher when microsomes of rats that were pretreated with phenobarbitone were tested. This means that phenobarbitone inducable enzymes play a role in the metabolization of these test compounds. Two pesticides that are able to form hemoglobin adducts *in vivo* were not able to affect the GT concentration in incubations with GSH solution, hemolysate or erythrocytes irrespective of the addition of MAS to the incubations. Hepatocytes imitate the *in vivo* situation more closely than MAS. For this reason, co-incubations of erythrocytes and hepatocytes were performed. Hepatocytes were able to metabolize CP and 3-HAA into GSH-reactive metabolites. Moreover, GSH-reactive metabolites of CP and 3-HAA were also excreted by the hepatocytes. However, erythrocyte GT was not affected in co-incubations of erythrocytes and hepatocytes testing 3-HAA, which may be explained by a minor diffusion uptake rate of metabolites into the erythrocytes.

In *chapters 4 and 5* the *in vivo* situation was approached more closely by perfusion of an isolated rat liver with medium and substrate. Liver cell viability was affected when no oxygen carriers were added to the medium. However, despite the lack of oxygen carriers in the medium, dimethylacetamide (DMAc) was eliminated from the medium (elimination rate constant=0.019 ± 0.008 min^{-1}). The addition of human erythrocytes to the perfusion medium had a positive effect on liver cell viability, which might lead to an increase of DMAc metabolism. However, no significant increase in the elimination rate constant of DMAc was detected.

The techniques which were developed in the preceding experimental chapters were applied to hydroxylamine (HYAM) and three oximes (cyclohexanone oxime (CHO), acetaldoxime (AAO) and methyl-ethyl ketoxime (MEKO)) in *chapter 6*. HYAM is a strong oxidative compound and oxidizes hemoglobin, causes the formation of thiobarbituric acid reactive substances (TBARS), inactivates glutathione S-transferase (GST) and lowers the GT concentration in erythrocytes. Oximes were less reactive compared to HYAM both in incubations with erythrocytes and hemolysate. In incubations with hemolysate, all three oximes caused oxidation of hemoglobin and inactivation of GST. These effects were also found in incubations with erythrocytes with exception of inactivation of GST caused by MEKO. However, the effects were less pronounced which may be explained by a low intracellular substrate concentration. AAO was the only oxime that caused TBARS both in incubations with erythrocytes and hemolysate. GT depletion was the least sensitive parameter tested since only MEKO was able to deplete GT in a concentration dependent way and this was only true for experiments with

hemolysate. Because oximes cause oxidative effects in blood *in vivo* and oximes are suggested to be metabolized into HYAM, rat liver preparations were added to the incubations with erythrocytes. However, the effects found in erythrocytes after co-incubation of erythrocytes and microsomal activating system (MAS) or hepatocytes were not higher compared to incubations with erythrocytes alone. This was also true when erythrocytes and oximes were perfused through an isolated rat liver. It is concluded that liver metabolism is not of major importance for the hematotoxic effects of oximes.

Based on the results obtained in this thesis it can be concluded that erythrocyte parameters were affected by several compounds. Which biochemical parameter(s) was/were affected depended on the compound tested. The effects found were higher in incubations with hemolysate compared to incubations with erythrocytes. Hemolysate had metabolic activity since GSH-reactive metabolites were formed out of several test substrates. The same substrates did not affect the GT concentration in erythrocytes. GSH or GT depletion was a proper effect parameter when electrophilic or strong oxidative compounds had to be detected. Hemoglobin oxidation or GST activity changes were better parameters in case of weak oxidative compounds. Some compounds had to be metabolized by MAS before they affected erythrocyte parameters. Co-incubation of erythrocytes with liver preparations that matched the *in vivo* situation more closely than MAS (hepatocytes or a total liver), did not enlarge the effects found in erythrocytes compared to incubations with erythrocytes alone. This can be explained by detoxification of reactive metabolites in the hepatocyte and by limited excretion of reactive metabolites from the hepatocyte. Although perfusion of erythrocytes through a isolated liver did not increase the effects found in incubations with erythrocytes alone, erythrocytes on their turn played an important role in the maintenance of liver cell viability. In the incubations in which erythrocytes were co-incubated with liver tissue, only MAS affected erythrocyte parameters. Because of this it can be said that the sensitivity of the test systems decreased when the *in vivo* situation was approached more closely. This may be explained by the capacity of hepatocytes and the total liver to adapt to changing circumstances.

Hoofdstuk 9

Samenvatting

Bloed speelt een centrale rol in het lichaam vanwege zijn transportfunktie. Daarbij verzorgen de erytrocyten het vervoer van zuurstof naar de weefsels en van kooldioxide terug naar de longen. Als gevolg van hun centrale rol komen erytrocyten in nauw kontakt met zowel organen die kunnen dienen als porte d'entrée voor lichaamsvreemde stoffen (xenobiotica) als met organen die deze xenobiotica kunnen transformeren. Bovendien is de erytrocyt zelf in staat metabolieten te genereren. Om zich tegen toxische verbindingen of hun metabolieten te beschermen bevat de erytrocyt diverse beschermingsmechanismen die vaak zijn gebaseerd op biochemische reakties. Wanneer de stress waaraan een cel wordt blootgesteld zo groot is dat de toxische verbinding niet wordt gedetoxificeerd kan dat leiden tot schade aan de cel.

In de lever komen erytrocyten in nauw kontakt met de metabool zeer aktieve hepatocyten. Niet toxische stoffen kunnen na metabolisering door de lever worden omgevormd in stoffen die toxisch zijn voor de erytrocyten. In dit proefschrift wordt voornamelijk aandacht besteed aan het samenspel tussen erytrocyten en hepatocyten waarbij met name aandacht wordt besteed aan de bioaktiverende en inaktiverende kapaciteit van zowel het bloed als de lever. Om deze vraagstelling te kunnen onderzoeken werden een aantal *in vitro* methodieken ontwikkeld.

Glutathion speelt een rol bij de bescherming van erytrocyten tegen elektrofiele en oxidatieve stress. Uit de *hoofdstukken 2 en 3* kunnen we afleiden dat de koncentratie gereduceerd glutathion (GSH) in erytrocyten daalde wanneer de cellen werden blootgesteld aan sterk elektrofiele verbindingen (joodacetamide, N-ethylmaleimide en diethyl maleaat) die in staat zijn de erytrocyt membraan te passeren. Genoemde verbindingen waren ook in staat GSH te depleteren in inkubaties met GSH opgelost in buffer (GSH oplossing), hetgeen betekent dat ze in staat zijn GSH te depleteren zonder voorafgaande metabolisering. Uit de experimenten met hemolysaat kunnen we afleiden dat 3-hydroxyacetanilide (3-HAA) en 2-methylfuraan (2-MF) door het hemolysaat worden gemetaboliseerd tot GSH-reaktieve verbindingen. De koncentratie totaal glutathion ((GT) dit is de som van de GSH en de geoxideerde glutathion (GSSG) koncentratie) in het hemo-

lysaat daalde koncentratieafhankelijk terwijl de GT koncentratie in inkubaties met GSH oplossing niet werd beïnvloed. 3-HAA en 2-MF hadden geen effekt op het GT gehalte in erytrocyten, mogelijk als gevolg van een te lage intracellulaire substraat koncentratie. Omdat hemolysaat niet gelijk mag worden gesteld aan erytrocyten zonder celmembraan, mag het verschil in effekt tussen inkubaties met hemolysaat en erytrocyten niet alleen worden verklaard uit de afwezigheid van de celmembraan.

Stoffen die niet in staat zijn GT in erytrocyten te depleteren kunnen dit mogelijk wel na extracellulaire metabole aktivering. Zowel cyclophosphamide (CP) als 3-HAA als 2-MF waren in staat de GT koncentratie in erytrocyten te depleteren na metabole aktivering door middel van microsomaal aktiverend systeem (MAS). De GT depletie werd nog versterkt wanneer in de inkubaties gebruik werd gemaakt van MAS van ratten die waren voorbehandeld met fenobarbital. Dit wijst op de betrokkenheid van fenobarbital induceerbare enzymen bij het metabolisme van bovenstaande verbindingen. Omdat de metabolieten in staat waren de GT koncentratie in erytrocyten te depleteren, mag hieruit worden afgeleid dat ze de celmembraan kunnen passeren. Twee pesticiden waarvan bekend is dat ze *in vivo* hemoglobine addukten vormen hadden geen effekt op de GT koncentratie in inkubaties met GSH oplossing, hemolysaat of erytrocyten. Toevoeging van MAS aan de inkubaten had geen effekt. Hepatocyten benaderen de *in vivo* situatie meer dan MAS. Om deze reden werden tevens co-inkubaties van erytrocyten met hepatocyten uitgevoerd. Hepatocyten waren in staat GSH-reaktieve verbindingen te genereren uit CP en 3-HAA. Tevens werden er GSH-reaktieve CP en 3-HAA metabolieten door de hepatocyt uitgescheiden. In co-inkubaties van erytrocyten en hepatocyten met 3-HAA als teststof kon echter geen GT depletie in erytrocyten worden waargenomen, hetgeen mogelijk verklaard kan worden door een te geringe opnamesnelheid van de metabolieten in de erytrocyt.

In de *hoofdstukken 4 en 5* werd de *in vivo* situatie nog meer benaderd door een totale geïsoleerde lever te perfunderen met medium waaraan de teststof is toegevoegd. Perfusiemedium zonder zuurstofcarriers bleek de levensvatbaarheid van de levercellen ernstig aan te tasten. Dimethylacetamide (DMAc) werd ondanks het gebrek aan zuurstofcarriers in het perfusiemedium toch geëlimineerd met een eliminatiesnelheidskonstante van $0{,}019 \pm 0{,}008$ min^{-1}. Toevoeging van humane erytrocyten aan het medium had een positieve invloed op de levensvatbaarheid van de levercellen, hetgeen tot een verhoogde biotransformatie van DMAc zou kunnen leiden. Een signifikante verhoging van de eliminatiesnelheidskonstante van DMAc kon echter niet worden aangetoond.

In *hoofdstuk 6* werden de technieken die in de voorgaande experimentele hoofdstukken werden gebruikt toegepast op hydroxylamine (HYAM) en een drietal oximen (cyclohexanonoxime (CHO), acetaldoxime (AAO) en methyl-ethyl ketoxime (MEKO)). HYAM staat bekend als een sterk oxidatieve verbinding en

oxideert hemoglobine, induceert lipide peroxidatie (LP), inaktiveert glutathion S-transferase (GST) en verlaagt de GT koncentratie in erytrocyten. De oximes bleken veel minder reaktief te zijn dan HYAM zowel in inkubaties met erytrocyten als met hemolysaat. In inkubaties met hemolysaat veroorzaakten alle geteste oximen oxidatie van hemoglobine en inaktivering van GST. De effekten waren, met uitzondering van GST inaktivering door MEKO, ook zichtbaar in erytrocyten hoewel ze minder sterk waren. Dit kan het gevolg zijn van een te lage intracellulaire substraat koncentratie. Alleen AAO veroorzaakte LP, zowel in inkubaties met erytrocyten als met hemolysaat. GT depletie bleek de minst gevoelige parameter te zijn omdat alleen MEKO een duidelijke koncentratieafhankelijke GT depletie veroorzaakte, en dan nog alleen in inkubaties met hemolysaat. Omdat oximes *in vivo* oxidatieve effekten in bloed veroorzaken en omdat oximes mogelijk worden gemetaboliseerd tot HYAM, werden rattelever preparaten toegevoegd aan de inkubaties met erytrocyten. De grootte van de effekten die in inkubaties met alleen erytrocyten konden worden aangetoond, nam echter niet toe na co-inkubatie van erytrocyten met MAS of hepatocyten. Hetzelfde gold voor perfusie van een geïsoleerde rattelever met medium waaraan oximen en erytrocyten waren toegevoegd. Op basis van deze resultaten kan worden gesteld dat metabole omzetting van oximen door de lever geen belangrijke rol speelt in de hematotoxische effekten van oximen.

Op basis van de resultaten verkregen in dit proefschrift kan worden gesteld dat verschillende xenobiotica in staat zijn biochemische parameters in erytrocyten te beïnvloeden. Welke biochemische parameters veranderden als gevolg van expositie aan xenobiotica, was afhankelijk van de geteste stof. Vergeleken bij inkubaties met erytrocyten waren de effecten in inkubaties met hemolysaat groter. Hemolysaat beschikt tevens over metabole kapaciteit omdat een aantal substraten werden gemetaboliseerd tot GSH-reaktieve verbindingen. Dezelfde substraten hadden geen effekt op de GT koncentratie in erytrocyten. GSH en GT depletie waren goede effekt parameters wanneer het elektrofiele of sterk oxidatieve verbindingen betrof. Zwak oxidatieve verbindingen konden beter worden aangetoond door middel van hemoglobine oxidatie of aktiviteitsverandering van GST. Sommige verbindingen beïnvloedden de erytrocyt parameters pas na metabole aktivering met behulp van MAS. Gebruik van aktiverende leverpreparaten die de *in vivo* situatie beter benaderden dan MAS (hepatocyten of de geïsoleerde rattelever), vergrootten de effekten die werden gevonden in inkubaten met alleen erytrocyten niet. Dit kan worden verklaard door ontgifting van reaktieve metabolieten in de hepatocyt en door een geringe uitscheiding door de hepatocyt van reaktieve metabolieten. Ofschoon perfusie van erytrocyten door een geïsoleerde lever geen effekt had op de gemeten parameters in de erytrocyten, hadden erytrocyten wel een positieve invloed op de levensvatbaarheid van de levercellen. Omdat van alle testsystemen waarin gebruik werd gemaakt van co-inkubaties van erytrocyten met leverweefsel alleen MAS effekt had op de gemeten parameters in de erytrocyten, kan worden

gesteld dat de gevoeligheid van de testsystemen afneemt naarmate ze de *in vivo* situatie beter benaderen. Dit kan mogelijk verklaard worden door het vermogen tot adaptatie van hepatocyten en de totale lever.

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Curriculum vitae

Nicole Palmen werd geboren op 23 april 1964 te Susteren. In 1982 behaalde zij het diploma Atheneum-B aan het Bisschoppelijk College te Echt. Daarna studeerde zij Gezondheidswetenschappen aan de Rijksuniversiteit Limburg te Maastricht en specialiseerde zich in de afstudeerrichtingen Verplegingswetenschap en Biologische Gezondheidkunde, die zij afsloot met het behalen van de Doctoraaldiploma's in respectievelijk 1987 en 1988.

Van augustus 1988 tot augustus 1993 was zij als Assistent in Opleiding in dienst van de Rijksuniversiteit Limburg. Het onderzoek werd gestart bij de vakgroep Arbeidsgeneeskunde Milieugezondheidkunde en Toxicologie (hoofd: prof. dr. P.Th. Henderson), en vervolgd bij de vakgroep Farmacologie (hoofd: prof. dr. H.A.J. Struijker Boudier), sectie Toxicologie. De dagelijkse begeleiding was in handen van dr. ir. C.T.A. Evelo. Tijdens de promotieperiode volgde ze tevens een aantal cursussen in het kader van de post-doctorale opleiding Toxicologie.

Sinds maart 1994 is zij aangesteld als arbeidstoxicoloog en arbeidshygiënist bij de Arbodienst Limburg en volgt momenteel de cursus arbeidshygiëne aan de Hogeschool West-Brabant.

Lijst van Publicaties

Full papers:

Borm, P.J.A., Palmen, N.G.M., Engelen, J.J. and Buurman, W.A. (1988) Spontaneous and stimulated release of tumor necrosis factor-α (TNF) from blood monocytes of miners with coal worker's pneumoconiosis. Am. Rev. Resp. Dis. 138, 1589–1594.

Palmen, N.G.M., Evelo, C.T.A., Borm, P.J.A. and Henderson, P.Th. (1992) Influence of oxygen supply on liver condition and elimination of dimethylacetamide in the isolated perfused rat liver. Toxicol. Vitro 6, 357–365.

Evelo, C.T.A., Palmen, N.G.M., Artur, Y. and Janssen, G.M.E. (1992) Changes in blood glutathione concentrations, and in erythrocyte glutathione reductase and glutathione S-transferase activity after running training and after participation to contests. Eur. J. Appl. Physiol. 64, 354–358.

Palmen, N.G.M., Evelo, C.T.A., Borm, P.J.A. and Henderson, P.Th. (1993) Toxicokinetics of dimethylacetamide (DMAc) in rat isolated perfused liver. Hum. Exp. Toxicol. 12, 127–133.

Palmen, N.G.M. and Evelo, C.T.A. (1993) Glutathione depletion in human erythrocytes as indicator for microsomal activation of cyclophosphamide and 3-hydroxyacetanilide. Toxicology 84, 157–170.

Palmen, N.G.M. and Evelo, C.T.A. Glutathione depletion in human erythrocytes and rat liver; a study on the interplay between bio-activation and inactivation functions of liver and blood. Submitted for publication.

Palmen, N.G.M., Feijts, A.H.J.M.G. and Evelo, C.T.A. Oxidative effects in human erythrocytes caused by some oximes and hydroxylamine. Submitted for publication.

Report:

Evelo, C.T.A., Palmen, N.G.M., Borm, P.J.A., Schins, P.F.R., Zondag, R., Pisters, R. and Leummens, M. (1991) Fysiologisch gebaseerd farmacokinetiek model (PB-PK model) voor 1,3-butadieen. Rapport opgesteld in opdracht van DSM-Limburg bv, kunststoffendivisie (KDS), Geleen.

Abstracts

Palmen, N.G.M., Sesink, A.L.A., Henderson, P.Th. and Borm, P.J.A. (1989) Application of recirculating and single pass liver perfusions to study interactions between workplace agents and drugs. Pharmaceutisch Weekblad Sci. Ed. 11, suppl. J, J8.

Evelo, C.T.A., Palmen, N.G.M., Bell, O.H.H., Kant, Y. and Borm, P.J.A. (1990) Physiological simulation model for study of metabolic interactions: choice of simulation package. Pharmaceutisch Weekblad Sci. Ed. 12, suppl. B, B6.

Evelo C.T.A., Palmen, N.G.M., Bell, O.H.H., Kant, Y. and Borm, P.J.A. (1990) Physiologically simulation model performance and usability when integrated in different simultation packages. International workshop on pharmacokinetic modelling in occupational health. Leysin Zwitserland, march 4–8.

Palmen, N.G.M., Borm, P.J.A., Sesink, A.L.A. and Henderson, P.Th. (1990) Application of recirculating and single pass lever perfusion to study effects of combined exposure. Leysin Zwitserland, march 4–8.

Palmen, N.G.M., Borm, P.J.A., Sesink, A.L.A., Evelo, C.T.A. and Henderson, P.Th. (1990) Liver perfusion and physiological simulation to study the effect of combined exposure. Beltox second annual meeting. Leuven, march 31, p. 7.

Palmen, N.G.M., Evelo, C.T.A. and Henderson, P.Th. (1991) Depletion of erythrocyte glutathione by extracellularly activated metabolites. Third international ISSX meeting, Amsterdam, june 24–28, Abstract no. 5A24.

Evelo, C.T.A., Palmen, N.G.M., ten Berge, W.F. and Borm, P.J.A. (1991) Physiologically based pharmacokinetic modelling of 1,3 butadiene: increased importance of lung metabolism at low doses. Third international ISSX meeting, Amsterdam, june 24–28, Abstract no. 9A10.

Palmen, N.G.M., Evelo, C.T.A., Borm, P.J.A. and Henderson, P.Th. (1991) Saturation of DMAc metabolism in isolated perfused rat liver. The 1991 Eurotox congress, Maastricht, september 1–4, Abstract no. 153.

Palmen, N.G.M., Evelo, C.T.A. and Henderson, P.Th. (1991) Depletion of erythrocyte glutathione by metabolites formed by fortified liver microsomes. The 1991 Eurotox congress, Maastricht, september 1–4, Abstract no. 154.

Evelo, C.T.A., Palmen, N.G.M., Pisters, R. and Borm, P.J.A. (1991) Development of a new software package for physiologically based pharmacokinetic modelling. The 1991 Eurotox congress, Maastricht, september 1–4, Abstract no. 243.

Evelo, C.T.A., Palmen, N.G.M., ten Berge, W.F. and Borm, P.J.A. (1991) Physiologically based kinetic modelling of 1,3 butadiene: importance of lung metabolism increased at low exposures. The 1991 Eurotox congress, Maastricht, september 1–4, Abstract no. 244.

Palmen, N.G.M., Evelo, C.T.A. and Henderson, P.Th. (1993) Glutathione depletion as an indicator of reactive metabolite formation in microsomes. Hum. Exp. Toxicol. 12, 71–72.

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